ANA RITA VIEIRA PINHEIRO

THE PURINOME IN FIBROBLASTS OF THE HUMAN SUBCUTANEOUS TISSUE

A CONTRIBUTION TO ELUCIDATE THE PATHOGENESIS OF MYOFASCIAL PAIN

Dissertação de Candidatura ao grau de doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Professor Doutor Paulo Jorge Silva Correia-de-Sá

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Dissertation in fulfillment of the requirements for the degree of Doctor in Biomedical Sciences, submitted to Instituto de Ciências Biomédicas Abel Salazar of the University of Porto.

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"If you want to have good ideas
you must have many ideas.
Most of them will be wrong,
and what you have to learn
is which ones to throw away."

Linus Carl Pauling
Nobel Prize in Chemistry, 1954
Nobel Peace Prize, 1962

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ABBREVIATIONS

$[Ca^{2+}]_i$, Intracellular calcium

8-PT, 8-Phenyltheophylline

a.u., Arbitrary units

ABC transporters, ATP-binding cassete transporters

AC, Adenylyl cyclase

ACP, Acid phosphatases

ADA, Adenosine deaminase

ADO, Adenosine

ADP, Adenosine 5'-diphosphate

AK, Adenylate kinase

ALP, Alkaline phosphatases

AMP, Adenosine 5'-monophosphate

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

ATP, Adenosine 5'-triphosphate

ATPγS, Adenosine 5′-[γ-thio]triphosphate tetralithium salt

Baf A1, Bafilomycin A1

BCA, Bicinchoninic acid

BFA, Brefeldin A

BK, Bradykinin

BSA, Bovine serum albumin

cAMP, Cyclic AMP

CBX, Carbenoxolone

CD39, Apyrase or NTPDase1

CD39L1, NTPDase2

CD39L3, HB6 or NTPDase3

CD73, Ecto-5'-nucleotidase

CFTR, Cystic fibrosis transmembrane conductance regulator

CGRP, Calcitonin gene-related peptide

CGS 21680, 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofu-ranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzene-propanoic acid hydrochloride

CHO, Chinese hamster ovary

CNT, Concentrative nucleoside transporters

COS-7, Fibroblast-like cell line derived from monkey kidney tissue

Cx, Connexin

DMEM, Dulbecco's Modified Eagle Medium

DMSO, Dimethylsulphoxide

DRG, Dorsal root ganglia

ECM, Extracellular matrix

EDTA, Ethylenediaminetetraacetic acid

EGTA, Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid

E-NPP, Ecto-nucleotide pyrophosphatase/phosphodiesterase

E-NTPDase, Ecto-nucleoside triphosphate diphospho-hydrolase

ENT, Equilibrative nucleoside transporters

ER, Endoplasmic reticulum

ERK, Extracellular signal-regulated kinase

FBS, Fetal bovine serum

FITC, Fluorescein isothiocyanate

GPCR, G-protein-coupled receptor

H1152, (*S*)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-hexahydro-1*H*-1,4-diazepine dihydrochloride

HBSS, Hank's Balanced Salt Solution

HEK-293, Human embryonic kidney 293 cells

HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

Hist, Histamine

HPLC, High-performance liquid chromatography

HRP, Horseradish peroxidase

HX, Hypoxanthine

IMP, Inosine monophosphate

INO, Inosine

IP_3, Inositol trisphophate

JNK, c-Jun N-terminal kinase

LDH, Lactate dehydrogenase

MAPK, Mitogen-activated protein kinases

MDLK, Madin-Darby canine kidney cells

MFQ, Mefloquine

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

MTT, Thiazolyl blue formazan, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan

MTX, Methrotrexate

NDP, Nucleoside diphosphate

NDPK, ectokinase

NECA, 5′-(N-ethylcarboxamide) adenosine, (2S,3S,4R,5R)-5-(6-aminopurin-9-yl)-N-ethyl-3,4-dihydroxyoxolane-2-carboxamide

NLRP3, NOD-like receptor family, pyrin domain containing 3

NMDA, N-methyl-D-aspartate receptor

NMP, Nucleoside monophosphate

NO, Nitric oxide

nRNA, Messenger ribonucleic acid

NTP, Nucleoside triphosphate

NTPDase, Nucleoside triphosphate diphosphohydrolase

PAGE, Polyacrylamide gel electrophoresis

Panx, Pannexin

PBS, Phosphate buffered saline system

PFA, Paraformaldehyde

PGP, Protein gene product

P_i, inorganic phosphate

PLC, Phospholipase C

PNP, Purine nucleoside phosphorylase

POM-1, Sodium metatungstate

PPADS, 4-[[4-Formyl-5-hydroxyl-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid tetrasodium salt

PVDF, Polyvinyl difluoride

RB-2, Reactive blue 2

RLU, Relative luminescence units

S.E.M., Standard error of the mean

SAPK, Stress-activated protein kinases

SCH 442416, 2-(2-Furanyl)-7-[3-(4-methoxyphenyl-)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]p-yrimidin-5-amine

SDS, Sodium dodecyl sulfate

SMA, α-Smooth muscle actin

SP, Substance P

$t_{1/2}$, Half-life time

TBS, Tris-buffered saline

TGFβ-1, Transforming growth factor β1

TH, Tyrosine hydroxylase

TNFα, Tumor necrosis factor α

TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) salt

U73122, 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione

UDP, Uridine 5'-diphosphate

UTP, Uridine 5'-triphosphate

VSOR, Volume-sensitive outwardly rectifying

WB, Western blot

RESUMO

Apesar do seu tamanho e localização ao longo de todo o corpo, o tecido conjuntivo não especializado tem sido geralmente ignorado ou mal interpretado, sendo considerado como relativamente supérfluo para além de desempenhar um papel de suporte dos tecidos mais especializados. Evidências recentes sugerem, no entanto, que o tecido conjuntivo subcutâneo pode tornar-se mais espesso e menos flexível em doentes com dor crónica, possivelmente como resultado de inflamação crónica e fibrose. Desta forma, a resposta esperada a um estiramento mecânico pode ser perturbada pela alteração das propriedades viscoelásticas do tecido conjuntivo subcutâneo, como consequência da remodelação mediada pelos fibroblastos e promovida por mediadores inflamatórios, como a histamina e a bradicinina. Considerando que o tecido conjuntivo subcutâneo é ricamente inervado por terminações nervosas sensoriais, as aferências de um tecido conjuntivo afetado podem alterar a percepção da dor.

Estudos anteriores demonstraram que a histamina e a bradicinina podem induzir a libertação de ATP a partir de vários tipos de células, incluindo células musculares lisas, células epiteliais, células endoteliais e uroteliais, e várias linhas de células imortalizadas. No entanto, os mecanismos de libertação de ATP induzidos pela bradicinina e pela histamina são ainda mal compreendidos, particularmente em tecidos humanos. Além disso, células danificadas durante a lesão dos tecidos moles podem provocar a libertação de grandes quantidades de ATP para o meio extracelular. Uma vez libertado, o ATP pode actuar como um mediador autócrino ou parácrino em células vizinhas, através da activação de receptores purinérgicos. Esta via de sinalização pode ainda ser modulada por ectonucleotidases ligadas à membrana, que sequencialmente catabolizem nucleósidos 5'-trifosfatos para o respectivo 5'-di- e monofosfato.

Considerando que [1] alterações na regulação da sinalização mediada pelo ATP no tecido conjuntivo pode ser importante na patogénese da dor inflamatória crônica e que [2] mediadores inflamatórios algogénicos, como a bradicinina e a histamina, podem sensibilizar as células de forma autócrina e parácrina por intermédio de sinais operados pelos nucleótidos de adenina extracelulares, este estudo pretendeu averiguar em fibroblastos isolados do tecido subcutâneo humano em cultura: 1) o envolvimento do ATP nas oscilações do cálcio intracelular produzidas pela bradicinina e pela histamina; 2) os mecanismos de libertação de ATP desencadeados pelos dois mediadores inflamatórios; 3) as vias

enzimáticas envolvidas no catabolismo extracelular do ATP e na formação de adenosina nestas células; e 4) a expressão dos receptores purinérgicos (P1 e P2).

Os resultados mostraram que a incubação tanto com histamina (100 μM) como com bradicinina (> 1 μM) aumenta a concentração citosólica de Ca^{2+} ($[Ca^{2+}]_i$) nos fibroblastos subcutâneos humanos por activação de receptores H_1 e B_2, respectivamente. Ambas as respostas apresentavam características bifásicas, tendo-se observado um aumento inicial rápido de Ca^{2+} que diminuiu rapidamente para um patamar sustentado acima da linha de base. O aumento do $[Ca^{2+}]_i$ induzido pela histamina e pela bradicinina parece depender da ativação da fosfolipase C (PLC) e do recrutamento de Ca^{2+} a partir das reservas intracelulares, o que é compatível com o envolvimento da via $G_q \rightarrow$ PLC $\rightarrow$ via IP_3, sinalização essa que é característica da activação dos receptores H_1 e B_2. Após a subida inicial transitória do Ca^{2+} devido ao recrutamento do Ca^{2+} do retículo endoplasmático, a fase de patamar foi mantida através da entrada de Ca^{2+} proveniente do meio extracelular. Paralelamente às oscilações de $[Ca^{2+}]_i$ observadas, estas células também libertaram ATP quando expostas aos dois mediadores inflamatórios, histamina e bradicinina. No entanto, os mecanismos envolvidos na libertação de ATP parecem ter características distintas. Embora os dois mediadores inflamatórios pareçam despoletar a abertura de hemicanais contendo panexina 1 (Panx1), o efeito da bradicinina parece envolver também hemicanais contendo conexinas (como a Cx43 e outras, possivelmente). Em ambas as situações, o ATP libertado desempenha um efeito the amplificação da resposta de $[Ca^{2+}]_i$ motivada pela activação de receptores P2. Os dados também mostraram que o patamar de elevação de $[Ca^{2+}]_i$ observada em células estimuladas com bradicinina foi, pelo menos em parte, atribuído à activação de receptores purinérgicos $P2Y_{12}$. Além disso, a exposição prolongada (até 28 dias) das células à histamina potenciou a sua proliferação, um efeito que foi parcialmente devido à activação do receptor $P2Y_1$.

Os resultados também mostram que, uma vez no meio extracelular, o ATP (3/30 μM) e o ADP (3/30 μM) foram metabolizados numa proporção global de aproximadamente 2 (ATP):1 (ADP), compatível com o envolvimento preferencial da E-NTPDase1 nas culturas de fibroblastos humanos de tecido subcutâneo. Este facto foi confirmado através de uma imunorreactividade mais evidente para a N-NTPDase1 comparativamente à E-NTPDase2; nestas células não se observou maracação positiva para a E-NTPDase3. Curiosamente, o AMP foi rapidamente desfosforilado em adenosina independentemente do substrato nucleotídico

utilizado (tempo de semi-vida de 1,5-3,0 min para concentrações de substrato de 3-30 μM, respectivamente). A existência de uma forte imunorreactividade para a ecto-5'nucleotidase / CD73 nestas células é compatível com os valores encontrados para as cinéticas enzimáticas e explica por que razão se observou uma acumulação negligenciável de AMP nas culturas, independentemente do nucleótido testado. Por outro lado, verificámos que os fibroblastos do tecido subcutâneo humano possuem uma fraca actividade da adenosina desaminase (ADA), metabolizando muito lentamente a adenosina (tempo de semi-vida de ~ 40-160 min, quando se incubaram as células com 3-30 μM de adenosina, respectivamente). Resultados preliminares sugerem ainda que a adenosina e os seus análogos estáveis potenciam a proliferação celular e a produção de colagénio tipo I pelos fibroblastos humanos em cultura, um efeito que parece ser devido à activação do receptor A_{2A}.

Concluindo, os fibroblastos do tecido subcutâneo humano expressam receptores purinérgicos funcionais, nomeadamente receptores $P2Y_1$, $P2Y_{12}$ e A_{2A}, bem como enzimas envolvidas na cascata de metabolização extracelular das purinas, nomeadamente as E-NTPDases1 e -2 e a CD73. Tanto a histamina como a bradicinina estimulam a libertação de ATP pelos fibroblastos humanos em cultura amplificando as suas respostas iniciais. Os mecanismos de sinalização envolvidos na resposta a cada mediador inflamatório parecem depender de microdomínios específicos nas células, nomeadamente no que respeita às vias de libertação do ATP (hemicanais do tipo Panx1 e Cx43, entre outras conexinas) e aos receptores purinérgicos ($P2Y_1$ e $P2Y_{12}$) envolvidos. Estes mecanismos podem envolver a mobilização dinâmica de ectonucleotidases (provavelmente a E-NTPDase2) para regiões específicas da membrana celular condicionando a resposta purinérgica mediante a acumulação (transitória) the determinada espécie nucleotídica, ATP ou ADP. Além disso, nestas células a adenosina tende a acumular-se no fluido extracelular e parece aumentar a proliferação, através da activação do receptor A_{2A}.

Assim, clarificar as vias que levam à libertação de nucleótidos e posterior sinalização purinérgica em fibroblastos humanos do tecido conjuntivo subcutâneo pode ser útil para: 1) compreender o papel deste tecido na patogénese de doenças músculo-esqueléticas dolorosas crónicas, 2) esclarecer os mecanismos que podem ser envolvidos nos efeitos analgésicos induzidos por manipulações de tecidos moles periféricos, tais como as técnicas de fisioterapia, e 3) formular estratégias terapêuticas farmacológicas inovadoras em relação à

modulação da comunicação entre as células inflamatórias, os fibroblastos e as terminações nervosas sensoriais.

ABSTRACT

Despite its overwhelming size throughout the body, unspecialized connective tissue has been generally overlooked or misunderstood, being considered as relatively superfluous apart from its supporting role amongst more specialized tissues. Recent evidences suggest, however, that subcutaneous connective tissue may become thicker and less compliant in patients with chronic pain, possibly as a result of chronic inflammation and fibrosis. Therefore, the normal response to mechanical stretch may be dampened by disturbance of the viscoelastic properties of subcutaneous connective tissue as a consequence of fibroblast remodeling promoted by inflammatory mediators, like histamine and bradykinin. Given that subcutaneous connective tissue is richly innervated by sensory nerve endings, inputs arising from affected connective tissue may alter pain perception.

Previous studies demonstrated that histamine and bradykinin elicit the release of ATP from various cell types, including smooth muscle fibers, epithelial, endothelial and urothelial cells, and several immortalized cell lines. Still, the mechanism of ATP release induced by bradykinin and histamine is poorly understood, particularly in human tissues. In addition, huge amounts of extracellular ATP may also leak from damaged cells during mild tissue injury. Furthermore, once released, ATP may act as an autocrine or paracrine mediator in neighboring cells via purinoceptors activation, a signaling pathway that may be modulated by membrane-bound ectonucleotidases, which sequentially catabolize nucleoside 5'-triphosphates to their respective 5'-di- and monophosphates.

Therefore, taking into consideration that [1] changes in the regulation of connective tissue ATP signaling may be important in the pathogenesis of chronic inflammatory pain and that [2] algogenic inflammatory mediators, such as bradykinin and histamine, may sensitize cells to autocrine and paracrine signals operated by extracellular adenine nucleotides, we investigated: 1) the involvement of ATP in bradykinin- and histamine-induced Ca^{2+} signals in human subcutaneous fibroblasts; 2) the ATP-release mechanisms triggered by bradykinin and histamine in human subcutaneous fibroblasts; 3) the extracellular enzymatic pathways responsible for ATP catabolism and adenosine formation in human subcutaneous fibroblast cultures; and 4) the expression of purinoceptors (P1 and P2) in fibroblasts from the human subcutaneous connective tissue.

Results showed that incubation with histamine (100 μM) and bradykinin (> 1 μM) increased cytosolic intracellular Ca^{2+} ($[Ca^{2+}]_i$) in human subcutaneous fibroblasts via the activation of H_1 and B_2 receptors, respectively. $[Ca^{2+}]_i$ responses were biphasic, as we observed a fast Ca^{2+} rise that declined rapidly to a sustained plateau above baseline. Histamine- and bradykinin-induced $[Ca^{2+}]_i$ rises depended on PLC activation and the recruitment of Ca^{2+} from intracellular stores, which is compatible with the involvement of the $G_q \rightarrow PLC \rightarrow IP_3$ signaling pathway that is characteristic of H_1 and B_2 receptors activation. Following the initial Ca^{2+} transient due to Ca^{2+} recruitment from the endoplasmic reticulum, the plateau phase was maintained via Ca^{2+} entry into human subcutaneous fibroblasts from the extracellular milieu. In parallel to the $[Ca^{2+}]_i$ oscillations observed, these cells also released ATP when exposed to histamine and bradykinin through different nucleotide-releasing pathways. While both histamine and bradykinin caused the opening of pannexin-1 (Panx1) hemichannels, connexin hemichannels (Cx43 and others, possibly) are only involved in bradykinin responses. For both inflammatory mediators, the released ATP amplified the $[Ca^{2+}]_i$ rise due to P2 receptors activation. Data also showed that the plateau of elevated $[Ca^{2+}]_i$ observed in cells stimulated with bradykinin was, at least in part, attributed to $P2Y_{12}$ purinoceptors activation. Moreover, prolonged exposure (until 28 days) to histamine potentiated the proliferation of fibroblasts, an effect that was partially due to the $P2Y_1$ receptor.

Results also demonstrated that, once in the extracellular milieu, ATP (3/30 μM) and ADP (3/30 μM) were metabolized with a ratio of approximately 2(ATP):1(ADP), which is compatible with E-NTPDase1 being the most effective isoform of E-NTPDases in these cultures. Furthermore, human subcutaneous fibroblasts showed a stronger immunoreactivity to E-NTPDase1 when compared to E-NTPDase2, and these cells presented no immunoreactivity against NTPDase3. Interestingly, AMP was rapidly dephosphorylated into adenosine independently of the nucleotide substrate used (half-life time of 1.5-3.0 min, 3-30 μM, respectively), and a strong immunoreactivity against ecto-5'nucleotidase/CD73 was also found. Conversely, adenosine deamination occurs slowly (half-life time of ~40-160 min, 3-30 μM, respectively), allowing for adenosine accumulation. Preliminary results also suggest that both proliferation and type I collagen production of cells continuously incubated with adenosine or its analogues increase, an effect that seems to be via the activation of the A_{2A} receptor.

Concluding, human subcutaneous fibroblasts express functional purinoceptors, such as $P2Y_1$ and $P2Y_{12}$, as well as the NTPDases1 and -2 and CD73. Both histamine and bradykinin stimulated human subcutaneous fibroblasts to release ATP to the extracellular milieu, which ultimately amplified the response induced by the primary ligand. For each mediator, the subsequent signaling might depend on specific microdomains of the cells, triggering a stimulus-specific rather than only a cell-specific response, involving different nucleotide-releasing pathways (Panx1 and Cx43 hemichannels, among other connexins) and purinoceptors ($P2Y_1$ and $P2Y_{12}$) activation, which might also involve the dynamic mobilization of ectonucleotidases (probably NTPDase2) to specific regions of the cell membrane. Moreover, in these cells adenosine tends to accumulate in the extracellular fluid leading to fibroblast cells growth and type I collagen production via A_{2A} receptor activation.

Thus, targeting the pathways leading to nucleotides release and subsequent purinergic signaling in human fibroblasts of subcutaneous connective tissue may be useful in: 1) understanding the role of this tissue in the pathogenesis of chronic painful musculoskeletal disorders; 2) clarifying the mechanisms that may be involved in the analgesic effects induced by peripheral soft tissue manipulations, such as physical therapy techniques; and 3) enlightening for novel pharmacological therapeutic strategies towards the modulation of the communication between inflammatory cells, fibroblasts and sensory nerve endings.

CHAPTER 1. INTRODUCTION

Nonspecialized connective tissue

The human body is mainly formed by four types of biological tissues, namely epithelial, muscular, nervous and connective tissues. The connective tissue plays several essential roles in the body, both structural, given that many of the extracellular elements possess special mechanical properties, and defensive, a role which is based on specific cellular functions. Often, it also has important trophic and morphogenetic roles in organizing and influencing the growth and differentiation of surrounding tissues.

The connective tissue can be divided in several subtypes, which can be generally grouped at large into specialized and nonspecialized tissues. All these tissues include two major components: cells and the extracellular matrix (ECM), which are specific according to the connective tissue subtype being considered (see Table 1.1). Specialized connective tissues consist of adipose tissue, cartilage, bone and blood. Nonspecialized connective tissues, which have long been termed by gross anatomists as fascia, embrace several undifferentiated mesenchymal tissues that wrap around what are sometimes regarded as the more 'specialized' organs and tissues of the body (1). Its major cell type, the fibroblast, regulates the ECM homeostasis by producing and degrading their constituents (2). In general, the ECM is mainly composed of glycoproteins and proteoglycans, many of which are able to bind to specific sites on other ECM glycoproteins so that the matrix becomes a highly crosslinked gel. There are approximately 100 known ECM components, more if one includes molecules such as growth factors that are not structurally part of the matrix, but which bind to it. In nonspecialized connective tissues, ECM is formed of ground substance, a watery gel rich in mucopolysaccharides or glycosaminoglycans such as hyaluronic acid, chondroitin sulfate, keratin sulfate, and heparin sulfate, and fibers of collagen, elastin and reticulin (3). Collagen, by far the most common protein in the body, that predominates in the fascial net is primarily of type I (4).

Table 1.1. Predominant structures of connective tissues.

Tissue type	Cells	Fibers	Interfibrillar elements, ground substance, water-binding proteins
Bone	Osteocyte, osteoblast, osteoclast	Collagen	Mineral salts, calcium carbonate, calcium phosphate
Cartilage	Chondrocyte	Collagen (and elastin)	Chondroitin sulfate
Fat	Adipocyte	Collagen	More proteoglycans
Blood	Red and white blood cells	Fibrinogen	Plasma
Dense connective tissues (ligaments, tendons, aponeurosis)	Fibroblast	Collagen	Minimal proteoglycans between fibers
Loose connective tissue	Fibroblasts, white blood cells, adipocytes, mast	Collagen and elastin	Significant proteoglycans

Adapted from (4).

In general, fascia is considered an uninterrupted viscoelastic tissue which forms a functional 3-dimensional collagen matrix that surrounds and penetrates all structures of the body extending from head to toes, binding some structures together, while permitting others to slide smoothly over each other, thus making it difficult to isolate and develop its nomenclature. Lack of consistent terminology has a negative effect on communication within health professions and compromises the advance of research (5). For example, depending on the authors, the connective tissue lamina of the hypodermis is named “textus connectives compactus”, “fascia superficialis”, “membranous layer”, “subcutaneous tissue”, “subcutaneous fascia” or “tela subcutanea” (1).

In 1983, the International Anatomical Nomenclature Committee confirmed the usage of previous nomenclature consensus and used the term “fascia superficialis” for the whole loose layer of subcutaneous tissue lying superficially to the denser layer of “fascia profunda”. While most health professionals in English speaking countries followed that terminology, other countries did not congruently adopt it. The subsequent international nomenclature, proposed by the Federative Committee on Anatomical Terminology in 1998, attempted to lead towards a more uniform international language and defined fascia as “…sheaths, sheets or other dissectible connective tissue aggregations”, including “investments of viscera and dissectible structures related to them”. This group suggested that authors should no longer use the term fascia for loose connective tissue layers and should apply it only to denser connective tissue aggregations (6). They gave a long list of terms related to their definition of fascia, and attempted to offer a system for grouping various fasciae based on embryological origins and modes of

development. However, clear details on the groupings and justification for this strategy were not given, remaining difficult to organize and properly use the multiple fascial terms (5). They also recommended the substitution of the old term "superficial fascia" for "tela subcutanea" or "subcutaneous tissue", besides suggesting the exclusion of the most frequently used "fasciae" names in anatomy from their proposed definition (5,6). This attempt, for the most part, failed; most English textbook authorities continued to use the term "superficial fascia" to describe subcutaneous loose connective tissues. In addition, an increasing number of non-English authors followed the British-American-trend in international medicine and started to adopt the same terminology as their American or British colleagues. In fact, according to the Gray's Anatomy, fascia consists of "…masses of connective tissue large enough to be visible to the unaided eye", "…fibres in fascia tend to be interwoven..." and includes "loose areolar connective tissue" such as the subcutaneous "superficial fascia" (6).

Concluding, discrepancies still exist concerning the official definition, terminology, classification and clinical significance of fascia (5). In an attempt to simplify, from now on we will consider the classification of superficial, deep (muscular) and visceral fascia, according to their anatomical location (6), in which subcutaneous connective tissue is considered part of the superficial fascia.

Despite its overwhelming relative size and relevance, superficial fascia has been heretofore generally overlooked or misunderstood as relatively superfluous by conventional dissection methods (1,7). The traditional idea of being a tissue for supporting specialized organs has the inherent and wrong implication that this tissue consists on less important supporting specialized tissues. Progressively, the errors of this assumption are being exposed and fascia is undoubtedly of considerable importance to many professionals working in health-related professions, like surgeons, physiotherapists, and others (1). In fact, 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 discrete and vital organ (7).

Sensory innervation of nonspecialized connective tissue

To date, little is known about the distribution and density of sensory nerve fibers in nonspecialized connective tissue. In 1957, Stilwell (8) identified numerous free nerve endings and large Pacinian corpuscles in the human thoracolumbar fascia. In 1963, Hirsch and coworkers (9) spoke of complex

unencapsulated endings without providing any illustrations. Later, in 1992, Yahia and coworkers (10) demonstrated the existence of two types of mechanoreceptors in the human thoracolumbar fascia, namely Ruffini's corpuscles (or Type I receptors) and Vater-Pacini corpuscles (or Type II receptors), as well as nerve bundles. Moreover, it has been reported that the thoracolumbar body region in rats is innervated by unmyelinated and myelinated afferents with conduction velocities in the range of C, Aδ and Aβ fibers and that the tissues supplied by these fibers are sensitive to mechanical and noxious chemical stimulation (11). Recently, in whole mount preparations, rat thoracolumbar fascia had the appearance of a densely innervated tissue exhibiting an extensive net of nerve fibers (11). The neuronal structures included fibers of passage and nerve endings characterized by chains of varicosities close to the nerve terminal. Fibers immunoreactive to the neuronal marker protein gene product (PGP) 9.5 were mainly found in the outer layer and subcutaneous tissue. A positive immunoreactivity against tyrosine hydroxylase (TH, characteristic of postganglionic sympathetic fibers), calcitonin gene-related peptide (CGRP) and substance P (SP), both peptides involved in nociception, was also found in the same regions. A quantitative evaluation of the fraction of CGRP- and SP-nerve fibers showed that they represent a small proportion of the total innervation visualized with the neuronal marker, PGP 9.5 (11). Conversely, another study demonstrated that, of the Fast Blue-labeled dorsal root ganglia (DRG) cells projecting from the nonspecialized connective tissues of the low back of rats, 60-88% expressed positive CGRP-immunoreactivity (12). A preliminary non-quantitative evaluation of the human thoracolumbar fascia (three patients) indicated that the innervation density of the human thoracolumbar fascia visualized by PGP 9.5 was likewise high and that the appearance of the free-nerve endings was similar. A high number of fibers showed TH-positive immunoreactivity indicating that the human fascia has a rich innervation with sympathetic fibers. Again, peptidergic fibers represented a small fraction of the total innervation. The stained neuronal structures included nerve endings characterized by chains of varicosities. The majority of peptidergic nerve endings were located in the subcutaneous tissue and in structures comparable with the outer and inner layer of the rat thoracolumbar fascia (11).

Together, these findings provide evidence that there are several types of sensory afferents in nonspecialized connective tissues, both mechanosensory and

nociceptive neurons, which may contribute to nociceptive circuitries, ultimately being involved in pain perception.

Role of nonspecialized connective tissues in musculoskeletal pain

Recent evidence suggests that nonspecialized connective tissues might be potential players in the pathophysiology and treatment of chronic pain conditions (13,14), such as low back pain, in a not well understood mechanism that could involve inflammation (15,16). In these studies, disorganization of the low back subcutaneous tissue, like thickness and fibrosis, was detected in patients with chronic low back pain (13,14).

One of the hallmarks of connective tissue is its plasticity in response to varying levels of mechanical stress. Both increased stress due to overuse, repetitive movement and/or hypermobility, and decreased stress due to immobilization or hypomobility, can cause changes in connective tissue, which in turn may have consequences in neighboring tissues. A chronic, local increase in stress can lead to microinjury and inflammation, and a consistent absence of stress, on the other hand, leads to connective tissue atrophy, architectural disorganization, fibrosis, adhesions and contractures. For example, during the early phase of immobilization, loss of muscle length is primarily due to the shortening of muscle-associated connective tissue (deep fascia), which is then followed by actual shortening of muscle fibers. Therefore, fibrosis can be the direct consequence of hypomobility or the indirect result of hypermobility via injury and inflammation (16). An altered tension due to the disorganization of richly innervated tissues may alter the sensory input arising from the affected tissues by making sensory neurons more responsive. Thus, smaller stimuli become capable of triggering a response. Furthermore, a sustained disorder of the nonspecialized connective tissue may induce a pathological neuroplastic rearrangement of its sensory innervation, thereby contributing to the development of a peripheral sensitization followed by a central sensitization.

Although it is unclear whether myofascial trigger points are the cause or effect of muscle injury, they are often exhibited in patients with musculoskeletal pain. They represent abnormally contracted muscle fibers, which 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 (17). Some of the signaling substances that accumulate nearby may activate nociceptors, besides the fact that myofascial

trigger points and muscle spasms may also contribute to connective tissue remodeling and fibrosis (18,19), In fact, inflammatory mediators, as well as other noxious chemical, mechanical and thermal stimuli, may influence nociceptors activity, therefore triggering nociceptive circuitries and pain (20,21). For example, bradykinin has been described as a potent algogenic substance by directly depolarizing nociceptors and by reducing their threshold (22). The role of histamine in nociception is not clear but it appears to cooperate with the effect of other endogenous mediators (23). ATP, acting on multiple purinergic receptors, either directly on neurons or indirectly through neural-glial cell interactions, seems to alter nociceptive sensitivity (24). Thus, inflammation might be directly involved in pain as well, due to the activation/modulation of nociceptive neurotransmission activity/threshold induced by inflammatory mediators.

Furthermore, activation of nociceptors can contribute to the development or worsening of fibrosis and inflammation, causing even more tissue stiffness and, as stated earlier, more pain. In the skin, it has been demonstrated that the release of SP from sensory C-fibers can enhance the production of histamine and cytokines from mast cells, monocytes and endothelial cells. Moreover, increased transforming growth factor β1 (TGFβ-1) production, stimulated by tissue injury and histamine release, seems to be a powerful driver of fibroblast collagen synthesis and tissue fibrosis (16).

Interestingly, recent evidence suggests that fibroblasts help to regulate the switch from acute resolving to chronic persistent inflammation (25). Until quite recently, fibroblasts were thought to predominantly provide physical structure to tissues by producing and remodeling the ECM of connective tissue. However, it has become increasingly clear that, in addition to their landscaping properties, fibroblasts play an active role in governing the persistence of inflammatory diseases (26) by guiding multiple cell types, such as mast cells, T cells, B cells, among others, through the release of signaling molecules, leading to their recruitment, retention, activation, and differentiation (27). However, their role in nonspecialized connective tissue-related musculoskeletal pain still needs clarification.

Finally, given that muscle activity is highly dependent on the compliance of the surrounding connective tissue, an altered tension due to disorganization of connective tissue will probably alter movement patterns. Additionally, both pain and pain-induced fear might cause altered activation patterns of postural muscles, resulting in dysfunctional movement patterns. Recent experiments

suggest that, in healthy individuals, fear of pain by itself can cause altered trunk muscle activation patterns during limb movement. Both experimental back pain (painful cutaneous electrical stimulation) and anticipation of pain (without electrical stimulation) caused increased activity and co-contraction of superficial muscles along with delayed or decreased activation of deep muscles. Thus, patients with chronic musculoskeletal pain appear to have a constellation of motion-limiting muscle activation patterns that may be initiated or aggravated by emotional factors (16)). At the end, dysfunctional movements might not provide an appropriate protection of the overused structures, which become more susceptible to injury (followed by inflammation and pain) (28).

Purinergic signaling dynamics - the "purinome"

Purines (adenosine, ADP and ATP) are molecules that are among the oldest and most influential biochemical compounds in the evolutionary history. In their physiologic state, mammalian cells contain high concentrations of ATP (5 to 8 mM) (29), which can be released in huge amounts by damaged cells and also from non-damaged cells under stressful conditions.

Nowadays it is well accepted that ATP, best known as the universal fuel inside living cells, also serves as a signal molecule that affects cell behavior (30). This 'purinergic signaling' hypothesis met considerable resistance in the beginning, partly perhaps because ATP was recognized at that time as an intracellular molecule contained in all cells and of particular importance as an energy source, and it was considered that such a ubiquitous molecule was unlikely to act as a neurotransmitter, even though the presence of powerful ectoenzymes for the extracellular breakdown of ATP were already known (31). Later, studies about the role of ATP as a messenger have increased evidence about how ATP signals work and why they are essential to basic bodily functions and development (30). In addition to the well-described physiological activities, extracellular nucleotides have been also recognized as having increasing importance in pathological conditions (32). Although ATP-signaling activity was first detected between nerve cells and muscle tissue, it is now known to operate within a wide variety of cell types in the body. In fact, because ATP is so ubiquitous, its influences can vary from tissue to tissue, offering new insights into a wide range of disorders and diverse ways to treat them (30).

In contrast to the compelling evidence for the extracellular signaling role of ATP, the hypothesis that UTP may also fulfill an autocrine/paracrine role has

only recently gained experimental support. In fact, the notion that pyrimidines (UDP and UTP) may function as extracellular messengers originated from the identification of a subset of P2Y receptors that recognize uridine nucleotides as their most potent and, in some cases, exclusive agonists (33).

Nucleoside- and nucleotide-releasing pathways

For many years, the source of extracellular nucleosides and nucleotides was considered to be damaged or dying cells, except for exocytotic vesicular release from excitable cells such as neurons. However, it is now known that several cell types release nucleosides and nucleotides constitutively and or in response to diverse physiological stimulation, such as shear stress (34,35). Most cells express transmembrane nucleoside transporters that can regulate the extracellular levels of these molecules, especially adenosine (35). The mechanisms of nucleotide transport to the extracellular media is currently being debated and includes the: (1) electrodiffusional movement through membrane ion channels, including connexin (Cx) and pannexin (Panx) hemichannels, stretch- and voltage-activated channels; (2) facilitated diffusion by nucleotide-specific ATP-binding cassette (ABC) transporters, such as the cystic fibrosis transmembrane conductance regulator (CFTR), the multidrug resistance proteins, and the multiple organic anion transporters; and (3) cargo-vesicle trafficking and exocytotic granule secretion (34-36).

Purinergic receptors

Purinergic effects are mediated via a series of nucleotide- and nucleoside-selective receptors, namely P2 and P1 receptors, respectively (36).

On the basis of their pharmacological and functional characteristics, two major receptor subfamilies of P2 receptors, P2X and P2Y, have been described. The P2X receptors are ligand-gated channels that gate extracellular cations in response to ATP and comprise seven receptor subtypes (P2X1-7) (see Table 1.2). The P2Y receptors are G-protein-coupled receptors (GPCR) that are categorized into a subfamily of receptors that predominantly couple to: (1) the G_q protein ($P2Y_{1,2,4,6,11}$), and therefore activate phospholipase C (PLC)-β; (2) the G_i protein ($P2Y_{12,13,14}$), thus inhibiting adenylyl cyclase (AC) and regulating ion channels (36); and (3) the G_s protein ($P2Y_{11}$), thus activating AC and regulating ion channels ($P2Y_{11}$ receptor is dually coupled to PLC and AC stimulation) (32).

Pharmacologically, P2Y receptors can be broadly subdivided into: (1) adenine nucleotide-preferring receptors, mainly responding to ADP and ATP; this group includes $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{11}$; (2) uracil nucleotide-preferring receptors; this group includes $P2Y_4$ and $P2Y_6$ receptors responding to either UTP or UDP; (3) receptors of mixed selectivity (human and rodent $P2Y_2$, rodent $P2Y_4$ and, possibly, $P2Y_{11}$); and (4) receptors responding solely to the sugar nucleotides UDP-glucose and UDP-galactose ($P2Y_{14}$) (36,37) (see Table 1.3).

Table 1.2. P2X ionotropic receptors.

Receptor	Endogenous ligand	Transduction mechanisms	Main agonists	Main antagonists
P2X1	ATP	Ion channel (Ca^{2+} and Na^{+})	ATP, α,β–MeATP	IP_5I, NF023, NF449, TNP-ATP
P2X2		Ion channel (mainly Ca^{2+})	ATP, ATPγS	isoPPADS, NF279, NF770, RB-2, suramin
P2X3		Intrinsic ion channel	ATP, 2-MeSATP	A317491, IP_5I, NF110, PPADS, TNP-ATP, RO-85
P2X4		Ion channel (mainly Ca^{2+})	ATP, α,β–MeATP	Coomassie blue, TNP-ATP
P2X5		Intrinsic ion channel (Cl^-)	ATP, α,β–MeATP	Coomassie blue, PPADS, suramin
P2X6		Intrinsic ion channel		-
P2X7		Intrinsic ion channel and a pore	ATP, BzATP	KN62, KN04, MRS2427, oATP

Adapted from (35).

Table 1.3. P2Y metabotropic receptors.

Receptor	Endogenous ligand	Primary transduction mechanisms	Secondary transduction mechanisms	Main agonists	Main antagonists
$P2Y_1$	ADP>ATP	$G_q/G_{11} \rightarrow$ PLC-β activation	G_i/G_o family → Ion channel (K^+ and Ca^{2+})	ADP, 2-MeSADP, ATP, ATPγS	2-ClATP, 2-MeSATP, MRS2179, MRS2279
$P2Y_2$	UTP=ATP	$G_q/G_{11} \rightarrow$ PLC-β activation	G_i/G_o family; G_{12}/G_{13} family	ATP, Ap_4A, UTP, UTPγS	ARC126312, suramin
$P2Y_4$	UTP>ATP	$G_q/G_{11} \rightarrow$ PLC-β activation	PLA_2 stimulation	ATP, Up_4U, Up_4dC, UTP	PPADS, RB-2, suramin
$P2Y_6$	UDP>>UTP>ATP	$G_q/G_{11} \rightarrow$ PLC-β activation	$G_{12}/G_{13} \rightarrow$ AC stimulation	MRS2693, UDP, UDPβS, Up_3U	MRS2567, MRS2575, MRS2578, RB-2
$P2Y_{11}$	ATP>UTP	$G_q/G_{11} \rightarrow$ PLC-β activation	$G_s \rightarrow$ AC stimulation	ARC67085, ATP, ATPγS, BzATP, dATP	5'AMPS, NF157, NF340, RB-2, suramin
$P2Y_{12}$	ADP>>ATP	$G_i/G_o \rightarrow$ AC inhibition	$G_i/G_o \rightarrow$ AC inhibition	ADP, ADPβS, 2-MeSADP, 2-MeSATP	AZD6140, BX667, Cangrelor, Clopidogrel, INS50589, AR-C66096
$P2Y_{13}$	ADP>>ATP	$G_i/G_o \rightarrow$ AC inhibition		ADP, ADPβS, 2-MeSADP, 2-MeSATP	Ap_4A, Cangrelor, 2-MeSAMP, MRS2211, MRS2603, RB-2
$P2Y_{14}$	UDP-glucose	$G_i/G_o \rightarrow$ AC inhibition	$G_i/G_o \rightarrow$ PLC stimulation	UDP, UDP-galactose, UDP-glucuronate, UDP-glucose	UDP

Adapted from IUPHAR and (35).

The P1 adenosine receptors are metabotropic receptors coupled to several families of G_i and G_o proteins, divided into four types of adenosine receptors ($A_{1,2A,2B,3}$) with distinct pharmacological and functional properties (see Table 1.4). As a rule, the A_1 and A_3 receptors exert an inhibitory effect on AC (mediated through $G_{i/o}$ proteins), whereas A_{2A} and A_{2B} receptors activate cyclic AMP (cAMP) production via G_s proteins. A_1 and A_3 receptors also regulate PLC and thus inositol 1,4,5-trisphosphate (IP_3) synthesis; in some cells, A_1 receptors were reported to activate K^+ and/or Ca^{2+} channels (38).

Table 1.4. P1 metabotropic receptors.

Receptor	Endogenous ligand	Primary transduction mechanisms	Secondary transduction mechanisms	Main agonists	Main antagonists
A_1	Adenosine	$G_i/G_o \rightarrow$ AC inhibition	Gs → AC stimulation; $G_i/G_o \rightarrow$ PLC stimulation	Adenosine, CCPA, R-PIA, NECA	DPCPX, CGS15943, CPT, FR194921, WRC-0571, XAC
A_{2A}		Gs → AC stimulation	$G_q/G_{11} \rightarrow$ AC stimulation and PLC activation	Adenosine, ATL146e, CGS21680, CV-1808, IAB-MECA, NECA	CGS15943, MRS1093, SCH58261, ZM241385
A_{2B}		Gs → AC stimulation	$G_q/G_{11} \rightarrow$ PLC activation	Adenosine, NECA	AS99, AS101, MRE2029F20, MRS1754, OSIP339391, XAC
A_3		$G_i/G_o \rightarrow$ AC inhibition	$G_i/G_o \rightarrow$ PLC stimulation	Adenosine, I-ABA, IAB-MECA, IB-MECA, NECA	MRS1177, MRS1220, MRE3010F20, VUF8504

Adapted from IUPHAR and (35).

Extracellular nucleoside and nucleotide metabolic pathways

Signal transduction mechanisms initiated by nucleotides are directly dependent on the extracellular levels of these messengers which, in turn, are modulated by the biochemical activity of ectonucleotidases (see Table 1.5). Ectonucleotidases are divided into two major categories: the nucleotide-degrading ectophosphohydrolases/esterases and the nucleotide-generating ectokinases/phosphotransferases (35).

Several ectonucleotidases have been described, including the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, acid (ACPs) and alkaline (ALPs) phosphatases, and ecto-5'-nucleotidase (CD73) (35,36). Of the 8 members of the NTPDase family (NTPDase1 to -8), only four are extracellular (E-NTPDase1-3 and -8). All E-NTPDases specifically hydrolyse tri- and diphosphonucleosides, albeit with different affinities (35), exclusively in the presence of divalent cations (Ca^{2+} or Mg^{2+}). E-NTPDase1 (also named CD39, ATPDase, ectoapyrase, ecto-ADPase) equally hydrolyzes ATP and ADP (ATP:ADP ratios of ≈1-2:1), E-NTPDase2 (ecto-ATPase, CD39Ll) is a preferential triphosphonucleosidase (ATP:ADP ratios of ≈10-40:1), and E-NTPDase3 (CD39L3, HB6) and E-NTPDase8 are functional intermediates between NTPDases 1 and 2 (ATP:ADP ratios of ≈3-4:1 and ≈2:1, respectively) (39). The E-NPP family comprises 7 ectoenzymes (NPP1 to -7) that possess the ability to hydrolyse a broad range of substrates present in the extracellular medium, including nucleoside tri- and diphosphates and their derivatives and/or lysophospholipids. NPP1 to -3 are the only members that can hydrolyse nucleotides such as ATP and ADP (35,40). The ACPs and ALPs families consist of several ectoenzymes that display broad substrate specificity towards different phosphomonoesters and other phosphated compounds, such as nucleotides (35,36). Of the 7 members of the 5'-nucleotidase family, only one is extracellular (ecto-5'-nucleotidase/CD73). CD73 catalyses the conversion of monophosphonucleosides to nucleosides and inorganic phosphate representing the main source of extracellular adenosine (35).

Once formed, adenosine and other nucleosides can be inactivated by ecto-adenosine deaminase (ecto-ADA) and/or by the cytosolic purine nucleoside phosphorylase (PNP) (36). Of the 3 members of the ADA family, only one is extracellular (ecto-ADA), which catalyses the irreversible deamination of

adenosine to inosine in the extracellular medium. Interestingly, analysis of the amino acid sequence of ecto-ADA shows no membrane-spanning domain nor membrane localization signal, suggesting that ecto-ADA is likely to be anchored to unidentified membrane proteins (35).

The nucleotide-generating ectokinases/phosphotransferases comprise the ecto-adenylate kinase (ecto-AK, also known as AK1β) and the ecto-nucleoside diphosphokinases (ecto-NDP kinases). Of the 8 members of the AK, only two are extracellular (ecto-AK). Ecto-AK catalyses the transfer of a phosphate group from triphosphonucleosides to nucleoside monophosphates, thus yielding two diphosphonucleosides in a reversible manner. The NDP kinase family is a group of 10 proteins (NM23-H1 to -H10), of which only two are membrane-bound (NM23-H1 and -H2). These enzymes transfer the phosphate groups from ATP to nucleoside diphosphates, thus generating ADP and triphosphonucleosides (35).

To support nucleotides-dependent signaling pathways, most cells need to recycle their building blocks (i.e. nucleobases or nucleosides) in a constant manner. To ensure that, two classes of membrane proteins, known as equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs), mediate their reuptake. ENTs are passive low-affinity facilitative transporters which allow nucleosides and, to a lesser extent, nucleobases, to diffuse across the plasma membrane according to their concentration gradient. In constrast, CNTs are high-affinity cation-dependent unidirectional symporters that use the potential energy built in the transmembrane Na^+ gradient to transfer extracellular nucleosides into the cytoplasm (35).

Often these ectonucleotidases work in concert or consecutively. ATP/UTP, for instance, can be hydrolyzed in one step to either ADP/UDP or AMP/UMP by E-NTPDase or NPP isoenzymes. ADP/UDP can be further hydrolyzed to AMP/UMP by E-NTPDases and NPPs, and AMP/UMP is converted to adenosine/uridine by alkaline phosphatases or the CD73. This sequential catabolism pathway not only terminates the triphosphonucleosides signaling but also generates intermediates with distinct signaling properties. Thus, diphosphonucleosides selectively interact with specific P2 receptor subtypes, while adenosine, acting through P1 receptors, plays its role as well. The relative contribution of the distinct ectonucleotidase species to the modulation of purinergic signaling may depend on differential tissue and cell distribution, regulation of expression, targeting to specific

membrane domains, but also on substrate availability and substrate preference (40).

Table 1.1. Potential ectoenzymes involved in the modulation of P2 and P1 purinoceptors signaling

Family	Ectoenzyme	Enzymatic reaction	Substrates	Functions or potential functions
NTPDases	NTPDase1 (CD39, apyrase, ATPDase)	NTP → NDP + Pi NDP → NMP + Pi	NTP ≈ NDP	Prevent $P2Y_1$ and P2X1 receptors desensitization; Terminate P2 receptors signaling ($P2Y_2$, P2Y6, P2X7, etc.); Favor adenosine generation
	NTPDase2 (CD39L1, ecto-ATPase)		NTP>>NDP	Termination of P2 receptors signaling; Switch of P2 receptors activation
	NTPDase3 (CD39L3, HB6)		NTP> NDP	Termination of P2 receptors signaling; Transient switch of P2 receptors activation
	NTPDase8 (Hepatic ATPDase)		NTP> NDP	Termination of P2 receptors signaling; Transient switch of P2 receptors activation
NPPs	NPP1 (CD203a, PC-1)	NTP → NMP + PPi NDP → NMP + Pi Np_nN → NMP + $Np_{(n-1)}$ NAD^+ → AMP + nicotinamide ribosyl-P 3'-5'- NMPc → NMP	ATP, Np_nN, pNP-TMP, NAD^+	Termination of P2 receptors signaling; Favor P2 receptors activation (from dinucleotides' hydrolysis); Favor adenosine formation
	NPP2 (autotaxin)		ATP, Np_nN, pNP-TMP, LPC, SPC	
	NPP3 (CD203c, $GP130^{RB13-6}$, B10)		ATP, Np_nN, pNP-TMP	
Phosphatases (ACP)	PAP (ACP3)	NMP → nucleoside + Pi	NMP, pNP-P, various phosphorylated molecules	Activation of P1 receptors
	OcAP/TrAP	NTP → NDP + Pi NDP → NMP + Pi NMP → nucleoside + Pi Molecule-P → molecule + Pi	NTP, NDP, NMP, pNP-P, various phosphorylated molecules	Termination of P2 receptors signaling; Activation of P1 receptors
Phosphatases (ALP)	TNAP (akp-2)	NTP → NDP + Pi NDP → NMP + Pi NMP → nucleoside + Pi Molecule-P → molecule + Pi	NTP, NDP, NMP, pNP-P, cAMP, various phosphorylated molecules	Termination of P2 receptors signaling; Activation of P1 receptors
Ecto-5'-nucleotidase	Ecto-5'-nucleotidase (CD73)	NMP → nucleoside + Pi	NMP	Activation of P1 receptors
Ectokinases	NDPK	NTP + N'DP ↔ NDP + N'TP	NTP, NDP	Swith of P2 receptors activation
	AK	2ADP ↔ AMP + ATP	ADP	Favor ADP-sensitive receptors ($P2Y_{1,12,13}$); Swith of P2 receptors activation
Ecto-ADA	Ecto-ADA (adenosine aminohydrolase)	Adenosine + H_2O → inosine + NH_4^+	Adenosine	Terminate/reduce P1 receptors signaling; Favor nucleobase uptake

Adapted from (39).

The purinome complex

As previously mentioned, it is now well described the existence of numerous different purinergic receptors sensitive to different endogenous ligands (ATP, UTP, ADP, UDP and adenosine), which couple to similar or quite different transduction pathways. On a first analysis, we might expect that

receptors that couple to the same intracellular signal transduction pathway would induce analogous cellular responses. However, the expression of similar or distinct membrane bound receptors and/or triggering of similar or distinct intracellular signal transduction pathways must not be considered as the unique criteria to explain what contributes to determine each specific cellular response to extracellular nucleotides (32). Recent evidence suggests that receptors, G proteins and effectors are specifically 3-dimensionally organized rather than freely diffused in the cell system, and that the specificity of signaling in intact cells appears to be significantly greater than in reconstituted systems (41). That indicates that nature is likely to be much more complex. From such a vast biological scenario and molecular complexity and diversity among purinergic receptors subunits, it clearly emerges that: (1) any purinergic receptors must be associated to a dynamic process; (2) multiple purinergic receptors are required for gaining biological functions; and (3) purinergic-related proteins are more than the sum of their single entities (32).

Increasing evidence demonstrates that the inner organization of the membrane of living cells plays a significant role in many biological processes and includes complex and distinctive biomolecular associative networks, able to ensure correct intracellular trafficking and fast communication among ligands, receptors, reuptake systems, degrading enzymes and signaling proteins (42,43). Therefore, biological compartments and submembrane microdomains at the plasmalemma level (lipid rafts, lipid rafts-like structures and caveolae) become critical elements in the coordination of receptor processes (42). Membrane rafts are defined as small (10-200 nm), heterogeneous, highly dynamic, cholesterol and sphingolipids containing microdomains that compartmentalize cellular processes. Under stimulating conditions, these smaller microdomains display a striking ability to coalesce and give rise to larger and stable membrane domains. Unlike planner membrane rafts, caveolae are a subset of raft characterized by 50-100 nm of flask-/omega-shaped membrane invaginations. Such invaginations have the advantage of being able to facilitate interactions between proteins that are localized in separate organelles (e.g. endoplasmic reticulum (ER) and mitochondria), thereby mediating a communication between separate membrane compartments (e.g. plasmatic membrane with ER) which would, otherwise, be several microns apart (43). These compartments set not only the biochemical entity and mechanisms of receptor cooperativity and functioning, but also the magnitude and direction (horizontal at the plasma membrane, vertical towards

the extra- and/or intracellular side of the cell) of the signals propagated by each ligand-activated receptor, and of the signals terminated by enzymatic degradation or reuptake systems. This large-scale synchrony/synergism of different molecular units, defined as the "receptosome", is then mechanistically ruled by specific receptor combinations activated within each cell phenotype, with the final aim of integrating the receptor assortment, to optimize the fulfillment of heterogeneous functions (42).

As an example of receptosome, the term "purinome" was coined to describe the molecular complex responsible for the biological effects of extracellular purine and pyrimidine ligands (42). In addition to a vast heterogeneity of purinergic ligands, the purinome thus consists of ectonucleotide-metabolizing enzymes hydrolysing nucleoside phosphates, P1 and P2 purinergic receptors, nucleoside transporters with both equilibrative and concentrative properties, and finally, nucleotide channels and transporters. Notably, these purinergic elements are not independent neither exists any recognized dominance subsisting among these proteins. Rather, all these proteins seem to play tightly concerted actions under physiological conditions. As a whole and not singularly, they trigger, maintain and terminate the purinergic signaling. This signifies that the purinome is not a new, mere definition of juxtaposed purinergic units, but rather the experimental evidence of complex and dynamic molecular cross-talk and cooperation networks. The alteration of their global and dynamic equilibrium might even be responsible for the appearance/ propagation of pathological states (42).

Purinergic signaling in inflammation and pain

Extracellular nucleotides and nucleosides, such as ATP and adenosine, have been identified as important signaling molecules that are involved in both inflammation (44) and pain (45) mechanisms.

During inflammatory conditions that occur in vascular thrombosis, hypoxia, ischemia, inflammatory bowel disease and acute lung injury, multiple cell types release nucleotides to the extracellular space, typically in the form of ATP and ADP. An additional source of extracellular nucleotides in such inflammatory conditions is provided by activated platelets, which release ATP and ADP through the release of granules and exocytosis. Once in the extracellular space, these nucleotides can exert their functions as signaling molecules. It seems that extracellular ATP has chemotactic effects on inflammatory cells and

activates the NLRP3 inflamassome. Examples of nucleotide-receptor signaling in inflammatory conditions include $P2Y_6$ or P2X7 receptors, which mediate vascular inflammation, $P2Y_1$, P2X1, and $P2Y_{12}$ receptors, which mediate platelet activation, and $P2Y_2$ and P2X7 receptors, which promote lung inflammation in chronic lung diseases such as asthma (29). There is, however, an often antagonistic and sometimes synergistic action between ATP and adenosine, which provides a mechanism for sophisticated cellular interactions. Therefore, ATP signaling should be considered together with adenosine signaling (46). In contrast to the ATP-induced pro-inflammatory effects, extracellular adenosine has been referred to as a "safety signal" that dampens inflammation. Experimental studies have shown that pharmacologic strategies that increase the breakdown of ATP to adenosine are effective in attenuating tissue injury and inflammation. For example in acute lung injury, it seems that enhanced inflammation that results from the activation of $P2Y_6$ and P2X7 receptors might be reverted by the activation of adenosine-sensitive receptors, possibly A_{2A} and A_{2B} receptors. Thus, shifting the balance between purinergic P2 and P1 signaling is an emerging therapeutic concept in efforts to dampen pathologic inflammation and promote healing (29).

Purinergic signaling seems to be an important regulatory mechanism in pain conditions as well. In 1996, Burnstock put forward a unifying purinergic hypothesis for the initiation of pain by ATP acting via P2X3 and P2X2/3 receptors. This hypothesis was first associated with causalgia, reflex sympathetic dystrophy, angina, migraine, and pelvic and cancer pain, but further research expanded this concept for acute, inflammatory, neuropathic, and visceral pain (45). Likewise, other purinergic receptors seem to be involved in nociceptive responses as well. The $P2Y_1$ receptor, which has been identified in a subpopulation of sensory neurons that also express the P2X3 receptor, is up-regulated in DRG neurons after transection of sciatic nerves, suggesting its involvement in nociception. Moreover, interactions between vanilloid and metabotropic P2Y receptors are being explored in terms of treatments for chronic pain and thermal hypersensitivity. Analgesic effects with intrathecal administration of the P2Y receptor agonists UTP and UDP in normal and the neuropathic pain rat model have also been reported, suggesting that $P2Y_2$ (and/or $P2Y_4$) and $P2Y_6$ receptors produce inhibitory effects in spinal pain transmission. Furthermore, P2X4 receptors expressed in spinal microglia appear to gate tactile allodynia after

nerve injury, and disruption of the P2X7 receptor gene abolishes chronic inflammatory and neuropathic pain (45).

In summary, there is increasing evidence demonstrating the expression of several P1, P2Y and P2X purinergic receptors in neurons, satellite glial cells, astrocytes, microglia and immune system cells, all of which, together with possible few more cell types, are involved in nociception (47). Altogether, these findings indicate that several purinergic receptors might evoke and/or modulate nociceptive mechanisms, both centrally and peripherally (46).

Purinergic signaling in specialized connective tissues

Bone

Bone development and remodeling comprise a complex purinergic signaling involving both P1 and P2 (P2X and P2Y) receptors, which are present in osteoclasts and osteoblasts (45). ATP and ADP, acting through P2X2 and $P2Y_1$ receptors, respectively, seem to turn on the activity of osteoclasts. On the other hand, ATP acting via the P2X5 receptor, has been reported to stimulate proliferation of osteoblasts, whilst activation of the $P2Y_1$ receptor is thought to modulate the response of osteoblasts to systemic factors, such as the parathyroid hormone. Though P2X7 receptor activation seems to reduce bone resorption by inducing apoptosis of osteoclasts, it has been also suggested that P2X7 receptor caused enhanced osteoblasts apoptosis. In contrast, P2X7 receptor stimulation in osteoblasts was associated to increased membrane blebbing and bone formation (48). Uracil nucleotides are also important regulators by mediating osteogenic cells differentiation, mainly through the activation of the UDP-sensitive $P2Y_6$ receptor. The endogenous actions of uracil nucleotides may be balanced through specific E-NTPDases, determining whether osteoblast progenitors are driven into proliferation or differentiation (49). Moreover, $P2Y_2$ receptor activation by ATP or UTP also seems to prevent mineralization by osteoblasts (48). Furthermore, adenosine is considered an important regulator of bone remodeling as well. A_{2B} receptor activation seems to have a consistent role in the differentiation of primary bone marrow stromal cells, which may be balanced through the relative strengths of A_1 or A_{2A} receptors determining whether osteoblasts are driven into proliferation or differentiation (50). Therefore, purinergic signaling might be an important therapeutic target in bone diseases, such as osteoporosis.

Cartilage

Similarly to the bone, purines play an important role in cartilage physiology. Mechanical stimulation, which influences the rate of nucleotides release and metabolism, is critical for the maintenance of healthy articular cartilage (45). However, evidence about purinergic signaling on cartilage seems to be controversial. Though it has been suggested that extracellular nucleotides, like ATP, negatively regulate cartilage metabolism due to the inhibition of cartilage formation, proteoglycan breakdown potentiation and increased production of inflammatory mediators, it has also been reported that ATP increases proteoglycan synthesis, collagen accumulation and suppression of the production of inflammatory mediators. In addition, ATP signaling, via P2X4 receptor activation, is thought to mediate the rise of intracellular Ca^{2+} required for chondrocyte differentiation (48). Moreover, it seems that nucleotide metabolism is tightly controlled by the cartilage extracellular matrix. Apart from modulating the activation of purinergic receptors, ectoenzymes regulate the levels of extracellular phosphate and pyrophosphate, which are the components involved in crystal deposition (45).

Furthermore, a purinergic signaling also seems to be involved in the pathophysiology of rheumatic diseases. For example, the articular fluid removed from arthritic joints contains increased levels of ATP, suggesting that ATP might potentiate the underlying inflammation levels of cartilage (45). In addition, methotrexate (MTX), which remains the most widely used agent for the treatment of rheumatoid arthritis and other chronic inflammatory diseases, exerts at least part of its anti-inflammatory effects via adenosine, possibly by altering the expression and function of adenosine receptors on inflammatory cells (51,52). Moreover, in MTX-resistant rheumatoid arthritis patients, blockage of the A_{2B} receptor has been shown an effective therapy and with minimal side effects in comparison to the anti-TNFα therapy, which is successful but accompanied by severe side effects (53). These findings are in agreement that, similarly to bone diseases, purinergic signaling might be an important therapeutic target in cartilage disorders.

Adipose tissue

There is evidence that nucleosides and nucleotides are important physiological regulators of adipose tissue function as well, whose dysfunction might ultimately contribute to obesity. Endogenous adenosine seems to promote

lipolysis (reviewed by (54)) and has been shown to increase the production of leptin in isolated white adipocytes through the A_1 receptor activation (reviewed by (55)). Though the mediating P2 purinergic receptors are still unidentified, as well as their functional significance, ATP and UTP were found to increase cytoplasmic calcium in white and brown adipocytes. In rat adipocytes, P2Y receptors were associated with lipogenesis and ATP was also found to decrease the release of leptin, possibly due to the $P2Y_{11}$ receptor activation (reviewed by (55)). On the other hand, $P2Y_1$ receptors can support leptin secretion in adipocytes and possibly affect the circulating levels of leptin in lean animals (55).

Blood

In the blood, extracellular nucleotides, such as ATP, ADP, UTP and UDP, are important signaling mediators that are strongly released by leucocytes, lymphocytes, endothelium, and platelets. Once released, these mediators bind to multiple P2Y and P2X receptors on platelets, endothelium, vascular smooth muscle cells, leucocytes and immunocytes, triggering short- and long-term processes affecting cellular metabolism, nitric oxide (NO) release, adhesion, activation and migration. As an example, the platelet activation and integrin binding necessary to platelet-aggregation seems to depend on the activation of $P2Y_1$, $P2Y_{12}$ and P2X1 receptors (56).

Purinergic signaling in nonspecialized connective tissues

Purinergic signaling in nonspecialized connective tissues is a ground to explore given that evidence is missing. This finding is not surprising due to the fact that nonspecialized connective tissues were considered less important, thus discarded until quite recently. The role of fibroblasts, its major cell type, has, however, been investigated in several organs, like lungs, intestine, liver and the heart (57-68).

In the intestine, fibroblasts seem to act as mechano-sensors, releasing ATP in response to mechanical stimulation, further eliciting a Ca^{2+}-wave propagation through the fibroblasts' network via $P2Y_1$ activation. The released ATP also seems to activate P2X receptors expressed in neighboring sensory neurons, regulating peristaltic motility (57).

Literature about cardiac fibroblasts as important myocardial remodeling key players after ischemic heart disease is also emerging and recent studies

suggest that purines and pyrimidines might be involved in this process. There is, however, some controversy. Although both ATP and UTP seem to induce a profibrotic response in mouse and rat cardiac fibroblasts by activating the $P2Y_2$ receptor (58,59), in rat *in vivo* models it has been described that UTP, via $P2Y_2$ receptor activation, induces cardioprotection by decreasing infarct size and restoring cardiac function following myocardial infarction (60,61). Furthermore, $P2Y_2$ receptor mRNA is up-regulated in patients with congestive heart failure, suggesting a pathophysiological role for this receptor in disease development (62). This controversy might be explained by the non-isolated cell-preparations, with which becomes unclear if results are attributable to cardiac fibroblasts, cardiomyocytes or endothelial cells. The expression of several other purinergic receptors in cardiac fibroblasts has also been reported, such as the $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptors, however their functional role remains to be clarified. In addition to the P2 receptors, P1 receptors also seem to play an important role in cardiac remodeling. Adenosine, via A_{2B} receptor activation, inhibits collagen production by cardiac fibroblasts (63,64), which might be considered a protective property for fibrosis development (63). Moreover, the downregulation of A_{2B} receptors seems to contribute to a disorganized collagen deposition and hypertrophy of cardiac fibroblasts following cardiac ischemia and reperfusion (65).

Conversely to what has been described in the heart, the activation of the A_{2B} receptor in the lungs seems to play a role in the pathogenesis of chronic obstructive pulmonary disease and interstitial fibrosis. Similarly, in the skin and in the liver, A_{2A} receptor activation seems to mediate and increase fibrosis due to an increase of collagen production, an effect that is prevented by the non-selective antagonist of P1 receptors, caffeine (66).

In pulmonary fibroblasts, little is known about the nature and the underlying mechanisms of purinergic responses in pulmonary fibroblasts, particularly in human, nor about their functional consequences. Recent data are consistent with extracellular nucleotides acting upon human pulmonary fibroblasts via the $P2Y_4$, $P2Y_6$ and/or $P2Y_{11}$ subtypes, rather than by $P2Y_1$ nor $P2Y_2$ subtypes, liberating internally sequestered Ca^{2+} leading to a Ca^{2+}-wave which courses throughout the cell and excites the nucleus, resulting in increased gene transcription of various proteins that are important for tissue repair and fibrosis (68).

Overall, these findings suggest that, according to their origin, differences in purinergic signaling cascades might occur in fibroblasts (see Table 1.6). This

finding is not unexpected. Differences in fibroblasts morphology have been mentioned for a long time, as well as in the proliferation potential between fibroblasts derived from specific regions (69)). Nevertheless, we are not aware of any studies which have characterized purinergic receptors and ectonucleotidases in human subcutaneous fibroblasts. Recently, it was demonstrated that rat subcutaneous fibroblast's cytoskeletal remodeling induced by tissue stretch involves ATP signaling (70), possibly due to the activation of $P2Y_1$, $P2Y_2$, $P2Y_4$ and/or P2X7 receptors (71). However, this is an open field still to be explored, especially when purinergic signaling may potentially play a role in connective tissue remodeling and, thereby, in the pathogenesis of connective tissue-related disorders.

Clinical relevance of this study

As previously mentioned, literature points out that subcutaneous connective tissue receives both proprioceptive and nociceptive innervation (10,12). Though the specific role of the identified nerve fibers remains unclear to consider this unspecialized connective tissue a sensory organ, such innervation supports recent studies that suggest the involvement of subcutaneous tissue in the pathophysiology of pain states, like back pain (13,14,16). This could occur either through direct activation or modulation of nociceptors activity or by its influence on muscle activity, therefore, in movement patterns (16).

Table 1.6. P2 purinergic receptors expressed in fibroblasts from different origins.

Origin	Species	Positive expression	Negative expression	Reference
Lung	Human	$P2Y_4$, $P2Y_6$, $P2Y_{11}$	$P2Y_1$, $P2Y_2$, P2X1, P2X3, P2X4, P2X6	(68)
Heart	Human	P2X4, P2X7, $P2Y_2$	P2X1, P2X2, P2X3, P2X5, P2X6, $P2Y_1$	(72)
Heart	Rat	$P2Y_2$		(59)
Heart	Rat	$P2Y_2$>>$P2Y_6$>>$P2Y_1$>$P2Y_4$	$P2Y_6$	(58)
Skin	Human	P2X7		(73)
Lung	Mouse	$P2Y_2$		(67)
Intestine	Rat	$P2Y_1$	$P2Y_6$, $P2Y_{12}$, P2X2, P2X7; $P2Y_4$?	(57)
Synovial tissue	Human	P2X1, P2X3, P2X7; lower expression of P2X2, P2X4, P2X5, P2X6		(74)
Subcutaneous tissue	Human	$P2Y_1$, $P2Y_2$, $P2Y_4$, P2X7	P2X1, P2X4	(71)

Interestingly, although the origins of astrocytes and fibroblasts are different (ectoderm and mesenchyme, respectively), both cells have very similar physiological characteristics and similar shape conversion mechanisms (75). Astrocytes, also classically considered as supportive cells, in this case for neurons, and without a direct role in brain information processing, are emerging as relevant elements in brain physiology through their ability to regulate neuronal activity and synaptic transmission and plasticity. In relation to the key role of astrocyte-neuron interactions in synaptic physiology, accumulating evidence suggests that dysfunctions of astrocyte-neuron signaling may be linked to the pathology of various neurological and neurodegenerative diseases (76). These findings support the idea that fibroblasts might also mediate and modify intercellular signal transductions among neighboring cells, like smooth muscles and neurons (75).

Increasing evidence suggests that purinergic signaling may potentially play a role in the function of fibroblasts and subsequent connective tissue remodeling, which could be the basis of the long-term pain-relieving benefits of acupuncture or physical therapy techniques involving mechanical manipulation of connective tissue (71). Thus, unveiling the purinergic mechanisms that underlie connective tissue remodeling might help to clarify their involvement in the pathogenesis of pain conditions, as well as to determine potential therapeutic targets for clinical use.

Taking into consideration that [1] changes in the regulation of connective tissue ATP signaling may be important in the pathogenesis of chronic inflammatory pain (44) and that [2] algogenic inflammatory mediators, such as bradykinin and histamine, may sensitize cells to autocrine and paracrine signals operated by extracellular adenine nucleotides (77), we investigated in this study:

- the involvement of ATP in bradykinin- and histamine-induced Ca^{2+} signals in human subcutaneous fibroblasts;

- the ATP-release mechanisms triggered by bradykinin and histamine in human subcutaneous fibroblasts;

- the extracellular enzymatic pathways responsible for ATP catabolism and adenosine formation in human subcutaneous fibroblast cultures;

- and the expression of purinoceptors (P1 and P2) in fibroblasts from human subcutaneous connective tissue.

CHAPTER 2. ORIGINAL RESEARCH PAPERS

PAPER 1

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Histamine induces ATP release from human subcutaneous fibroblasts via pannexin-1 hemichannels leading to Ca^{2+} mobilization and cell proliferation

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ABSTRACT

Changes in the regulation of connective tissue ATP-mediated mechano-transduction and remodeling may be an important link to the pathogenesis of chronic pain. It has been demonstrated that mast cell-derived histamine plays an important role in painful fibrosing diseases. Here we analyzed the involvement of ATP in the response of human subcutaneous fibroblasts to histamine. Acute histamine application caused a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and ATP release from human subcutaneous fibroblasts via H_1 receptor activation. Histamine-induced $[Ca^{2+}]_i$ rise was partially attenuated by apyrase, an enzyme that inactivates extracellular ATP, and by blocking P2 purinoceptors with 4-((4-formyl-5-hydroxyl-6-methyl-3-((phosphonooxy)-methyl)-2-pyridinyl)azo)-1,3-benzenedisulfonic acid tetrasodium salt and reactive blue 2. $[Ca^{2+}]_i$ accumulation caused by histamine was also reduced upon blocking pannexin-1 hemichannels with ^{10}Panx, probenecid or carbenoxolone, but not when connexin hemichannels were inhibited with mefloquine or 2-octanol. Brefeldin A, an inhibitor of vesicular exocytosis, also did not block histamine-induced $[Ca^{2+}]_i$ mobilization. Prolonged exposure of human subcutaneous fibroblast cultures to histamine favored cell growth and type I collagen synthesis via the activation of H_1 receptor. This effect was mimicked by ATP and its metabolite, ADP, whereas the selective $P2Y_1$ receptor antagonist, 2'-deoxy-N^6-methyladenosine 3',5'-bisphosphate tetrasodium salt, partially attenuated histamine-induced cell growth and type I collagen production. Expression of pannexin-1 and ADP-sensitive $P2Y_1$ receptor on human subcutaneous fibroblasts was confirmed by immunofluorescence confocal microscopy and Western blot analysis. In conclusion, histamine induces ATP release from human subcutaneous fibroblasts via pannexin-1 hemichannels leading to $[Ca^{2+}]_i$ mobilization and cell growth through the cooperation of H_1 and P2 (probably $P2Y_1$) receptors.

Keywords: human subcutaneous fibroblasts, ATP release, pannexin 1, P2 purinoceptors, histamine

INTRODUCTION

Unspecialized connective tissue forms an anatomical mesh throughout the body, which acts as a body-wide mechanosensitive signaling network (7,78). Despite its overwhelming size, the relative importance of unspecialized connective tissue has been generally overlooked or misunderstood; it was considered as relatively superfluous apart from its supporting role amongst more specialized tissues (7). Nowadays, evidence suggests that increased connective tissue disorganization may be an important link in the pathogenesis of chronic musculoskeletal pain, such as low back pain (14,16), and we hypothesized that this might also occur in fibromyalgia.

Like the skin, the underlying subcutaneous connective tissue is richly innervated by sensory nerve endings, including mechanoceptors, nociceptors and thermoceptors (12,79). Therefore, sensory inputs arising from affected connective tissue may alter the activity of nociceptors. Conversely, activation of nociceptors causes the release of substance P from sensory C-fibers, which triggers the production of inflammatory mediators, like histamine and several cytokines, from neighboring cells (80). It was demonstrated that substance P and histamine augment cytokine-induced proliferation of dermal fibroblasts, thus indicating that mast cell-derived histamine may be a key player in the induction of tissue fibrosis in painful fibrosing diseases (81). In view of this, nociceptor activation may, by itself, contribute to prolong inflammation and to exaggerate tissue fibrosis.

Inflammatory mediators, such as cytokines and histamine, are essential to the onset and maintenance of inflammatory reactions (82). In addition, adenine and uracil nucleotides have also been related to the pathophysiology of chronic inflammation (44). Besides their acute actions, nucleotides exert autocrine and/or paracrine activities regulating cellular processes, like proliferation and differentiation of human cells (49). Interestingly, it has been described that smooth muscle and epithelial cells release ATP in response to histamine (83), but this was never demonstrated in fibroblasts. Therefore, we postulated that inflammatory mediators, like histamine, could influence connective tissue plasticity through the release of ATP from fibroblasts, the predominant cell type of this tissue, leading to signal amplification via P2 purinoceptors located in neighboring cells.

Nucleotides reach the external milieu via both lytic and non-lytic mechanisms from various cell types upon mechanical or chemical stimulation.

Nucleotide-releasing pathways include [1] electrodiffusional movement through membrane ion channels, including pannexin and connexin hemichannels; [2] facilitated diffusion by nucleotide-specific ATP-binding cassete (ABC) transporters; and [3] cargo-vesicle trafficking and exocytotic granule secretion (36). The extent of the paracrine activity mediated by ATP and/or related released nucleotides may be limited by the presence of membrane-bound nucleotide metabolizing enzymes. Ectonucleotidases sequentially catabolize nucleoside 5'-triphosphates to their respective nucleoside 5'-di- and monophosphates, nucleosides and free phosphates or pyrophosphate, which can all appear in the extracellular fluid at the same time (36). Although a comprehensive study on the kinetics of extracellular catabolism of adenine nucleotides and adenosine formation in the human musculoskeletal system is still lacking, the coexistence of various metabolic pathways for the nucleotide extracellular hydrolysis represents an opportunity for regulating cell-specific responses to surrounding adenine nucleotides and for terminating purinergic actions.

Therefore, this study was designed to investigate if the response of human subcutaneous fibroblasts to histamine involves a purinergic loop consisting in the release of ATP, formation of its biological active metabolites (namely ADP) and subsequent P2 purinoceptors activation. Understanding the mechanisms underlying the purinergic amplification loop regulating cell signaling and subcutaneous tissue remodeling triggered by inflammatory mediators may highlight new therapeutic strategies for chronic painful conditions.

EXPERIMENTAL PROCEDURES

Cell cultures

Human fibroblasts were isolated from the subcutaneous tissue of organ donors (52 ± 5 years old (mean ± S.E. of the mean), n = 16) with no clinical history of connective tissue disorders. The protocol was approved by the Ethics Committee of Hospital Geral de Santo António SA (University Hospital) and of Instituto de Ciências Biomédicas de Abel Salazar (Medical School) of University of Porto. The investigation conforms to the principles outlined in the Declaration of Helsinki. Subcutaneous tissues were maintained at 4-6ºC in M-400 transplantation solution (4.190 g/100 mL mannitol, 0.205 g/100 mL KH_2PO_4, 0.970 g/100 mL $K_2HPO_4 \cdot 3H_2O$, 0.112 g/100 mL KCl, and 0.084 g/100 mL $NaHCO_3$, pH 7.4) until used, which was between 2 and 16 hours after being harvested (84). Cells were then obtained by the explant technique and cultured in DMEM medium

supplemented with 10% fetal bovine serum (FBS), 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 until near confluence (~3 – 4 weeks), then adherent cells were enzymatically released with 0.04% trypsin-EDTA solution plus 0.025% type I collagenase in phosphate-buffered saline (PBS). The resultant cell suspension was cultured and maintained in the same conditions mentioned above. All the experiments were performed in the first subculture.

Extracellular ATP quantification by bioluminescence

Extracellular ATP was quantified by the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science) using a multi detection microplate reader (Synergy HT, BioTek Instruments). Briefly, cells were seeded in flat bottom 96 well plates at a density of $3x10^4$ cells/mL for 21 days. At the beginning of the experiment, cells were washed twice with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 11.2 mM glucose, pH 7.4) at 37ºC and let rest during 30 minutes (basal), after which samples were collected (75 µL). Subsequently, histamine (100 µM) was added. Samples were collected at five different incubation times (0 – 240 seconds) and immediately stored at -20ºC. Luciferin-luciferase experiments were performed at room temperature and light emission acquisition was performed 20 seconds after addition of luciferin-luciferase to the collected sample.

Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were measured with the calcium sensitive dye Fluo-4 NW using the multi detection microplate reader referred above (85). Human fibroblasts were seeded in flat bottom 96 well plates at a density of $3x10^4$ cells/mL. Cells were cultured for 5 – 15 days in supplemented DMEM as described before. On the day of the experiment, cells were washed twice with Tyrode's solution and incubated at 37ºC for 45 minutes with the cell-permeant fluorescent Ca^{2+} indicator, Fluo-4 NW (2.5 µM). After removal of the fluorophore loading solution, cells were washed again twice and 150/300 µL of Tyrode's solution was added per culture well/dish, respectively. For the recordings, temperature was maintained at 32ºC and readings were made with 5 seconds of interval, during approximately 30 minutes, using a tungsten halogen lamp. Fluorescence was

excited at 485/20 nm and emission was measured at 528/20 nm. Calcium measurements were calibrated to the maximal calcium load produced by ionomycin (5 μM, 100% response) (86,87).

Single-cell $[Ca^{2+}]_i$ imaging and To-Pro3 dye uptake detection by confocal microscopy

In some of the experiments, we monitored single-cell $[Ca^{2+}]_i$ oscillations and To-Pro3 dye uptake from the same cells by confocal microscopy (FV1000, Olympus, Japan) (49). Due to their high molecular mass, carbocyanine monomer nucleic acid fluorescent dyes, like To-Pro3 (671 Da), are membrane impermeable and generally excluded from viable cells unless large diameter pores open. Thus, increases in fluorescence intensity can be taken as measure of To-Pro3 dye uptake into viable cells, which may occur through connexin and pannexin-1 hemichannels that conduct molecules up to 1 kDa in size across the plasma membrane (ATP has 507 Da). Human subcutaneous fibroblasts were seeded onto 35 mm glass bottom dishes at a density of $2x10^4$cells/mL and allowed to grow for 5 – 15 days in supplemented DMEM, as described before. On the day of the experiment, cells were first loaded with the fluorescent Ca^{2+} indicator, Fluo-4 NW (2.5 μM, see above). Culture dishes were mounted on a thermostatic (32ºC) perfusion chamber at the stage of an inverted laser-scanning confocal microscope equipped with a 20x magnification objective lens (LUCPLFLN 20x PH; NA: 0.45). The chamber was continuously superfused (1 mL/min) with gassed (95% O_2, 5% CO_2, pH 7.4) Tyrode's solution. To monitor histamine-induced hemichannels opening in parallel to $[Ca^{2+}]_i$ oscillations, To-Pro3 iodide (1 μM) was added to the superfusion solution 6 minutes before histamine application and was kept up to the end of the experiment. Fluo-4 NW was excited with the 488 nm Multi-line Ar laser and the emitted fluorescence was detected at 510-560 nm; TO-PRO-3 was excited with the 633 nm Red He-Ne laser and the emitted fluorescence was detected at 661 nm. Time-lapse sequences were recorded at scanning rates with 20 seconds of interval for approximately 30 minutes, digitized, and processed off-line. Regions of interest were defined manually.

Cell viability/proliferation

Viability /proliferation studies included the MTT assay as previously described (50). Human fibroblasts were seeded in flat bottom 96 well plates at a

density of $3x10^4$ cells/mL and cultured in supplemented DMEM as described before. Cell cultures were routinely monitored by phase contrast microscopy and characterized at days 1, 7, 14, 21 and 28. The 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 4 h of each test period, cells were incubated with 0.5 mg/mL of MTT for 4 h 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/well.

Total type I collagen determination

Type I collagen determination was performed using the Sirius Red staining assay. Human fibroblasts were cultured as described for the viability/proliferation studies. The staining protocol was adapted from Tullberg-Reinert & Jundt (1999) (88). Cell layers were washed twice in PBS before fixation with Bouin's fluid for 1 h. The fixation fluid was removed by suction and the culture plates were washed by immersion in running tap water for 15 minutes. Culture dishes were allowed to air dry before adding the Sirius Red dye (Direct Red 80). Cells were stained for 1 h under mild shaking on a microplate shaker. To remove non-bound dye, stained cells were washed with 0.01 N hydrochloric acid and then dissolved in 0.1 N sodium hydroxide for 30 minutes at room temperature using a microplate shaker. Optical density was measured at 550 nm against 0.1 N sodium hydroxide as blank (88). Results were expressed as A/well.

Enzymatic kinetic experiments and HPLC analysis

After a 30-min equilibration period at 37ºC, culture day 11 cells were incubated with 30 μM ATP, ADP or AMP, which were added to the culture medium at zero time. Samples (75 μL) were collected from each well at different times up to 30 minutes for high-performance liquid chromatography (HPLC, LaChrome Elite, Merck) analysis of the variation of substrate disappearance and product formation (49,89). ATP and ADP catabolism analysis was performed by ion-pair reverse-phase HPLC (90), whereas for AMP catabolism analysis was used a linear gradient (100% of 100 mM KH_2PO_4, pH = 7, to 100% of 100 mM KH_2PO_4, pH = 7, -30% of methanol) during 10 minutes with a constant rate flow of 1.25 ml/min. Under these conditions, the retention times of metabolites was as follows: AMP

(2.17 min), hypoxantine (3.07 min), inosine (5.09 min) and adenosine (7.51 min). Concentrations of the substrate and products were plotted as a function of time (progress curves). The following parameters were analyzed for each progress curve: half-life time ($t_{1/2}$) of the initial substrate, time of appearance of the different concentrations of the products, concentration of the substrate or any product remaining at the end of the experiment.

The spontaneous degradation of adenine nucleotides and nucleosides at 37ºC in the absence of the cells was negligible (0 – 3%) over 30 minutes. At the end of the experiments, the remaining incubation medium was collected and used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity. The negligible activity of LDH in the samples collected at the end of the experiments is an indication of the integrity of the cells during the experimental period.

Antibody production

The development and specificity of anti-human NTPDase2 and NTPDase3 has been reported previously (91,92). Hartley guinea pigs and New Zealand rabbits were obtained from Charles River Laboratories (Quebec City, Canada). Genetic immunization protocol was carried out with plasmids (pcDNA3 for human NTPDase1 and pcDNA3.1 for human CD73) encoding each protein using New Zealand rabbits for antibodies against human NTPDase1 and Hartley guinea pigs for human CD73 antibodies.

Immunocytochemistry

Human fibroblasts were seeded in chamber slides at a density of $2.5x10^4$ cells/mL and allowed to grow for 5 – 15 days. Cultured cells were fixed in 4% paraformaldehyde (PFA) in 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% NaN_3) for 1 h. Primary antibodies, diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X, 0.05% NaN_3), were applied [mouse anti-porcine vimentin 1:75 (DAKO); rabbit anti-human collagen I 1:50 (AbDSerotec); rabbit anti-human $P2Y_1$ 1:50, rabbit anti-human $P2Y_{12}$ 1:100 and rabbit anti-human $P2Y_{13}$ 1:25 (Alomone); rabbit anti-human Cx43 1:600 and rabbit anti-human Panx1 1:1000 (Abcam); rabbit anti-human NTPDase1 1:100, rabbit anti-human NTPDase2 1:200, mouse anti-human NTPDase3 1:200 and guinea-pig anti-human CD73 1:300 (University of Laval, Québec, QC, Canada; see

the Centre de Recherche du CHUL Web site for further details) and the slides incubated overnight at 4ºC. After incubation, cells were washed 3 times in PBS 1X (10 minutes each). The donkey anti-rabbit IgG Alexa Fluor 488, donkey anti-mouse IgG Alexa Fluor 488, donkey anti-mouse IgG Alexa Fluor 568 and donkey anti-guinea pig IgG Alexa Fluor 568 secondary antibodies (Invitrogen) 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 were mounted with VectaShield medium and stored at 4ºC. Observations were performed and analyzed with an Olympus FV1000 confocal microscope (49,93).

SDS-PAGE and Western blotting

Fibroblasts were homogenized in a lysis buffer with the following composition: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS and a protease inhibitor cocktail. Protein content of the samples was evaluated using the BCA protein assay kit according to the manufacturer's instructions (Pierce). Samples were solubilized in SDS reducing buffer (0.125 mM Tris-HCl, 4% SDS, 0.004% bromphenol blue, 20% glycerol, and 10% 2-mercaptoethanol, pH 6.8 at 70ºC for 10 minutes), subjected to electrophoresis in 10% SDS-polyacrylamide gels and electrotransferred onto PVDF membranes (MilliPore). Protein loads were 60 µg for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$, 25 µg for Panx1 and 15 µg for Cx43. The membranes were blocked for 1 h in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 + 5% BSA. Membranes were subsequently incubated with goat anti-human $P2Y_1$ 1:200 (Santa Cruz), rabbit anti-human $P2Y_{12}$ 1:200 (Alomone), rabbit anti-human $P2Y_{13}$ 1:200 (Alomone), rabbit anti-human Panx1 1:250 (Novex, Life Technologies) and rabbit anti-human Cx43 1:6000 (Abcam) in the above blocking buffer overnight at 4ºC. Membranes were washed three times for 10 minutes in 0.1% Tween 20 in TBS and then incubated with donkey anti-rabbit IgG (HRP) 1:30000 (Abcam) and donkey anti-goat IgG (HRP) 1:25000 (Abcam) secondary antibodies, for 60 minutes at room temperature. For comparison purpose, the membranes were also incubated with rabbit anti-human β-tubulin 1:2500 (Abcam) antibody following the procedures described above. Membranes were washed three times for 10 minutes and antigen-antibody complexes were visualized by chemiluminescence with an ECL reagent using the ChemiDoc MP imaging system (Bio-Rad Laboratories).

Materials and reagents

1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (thiazolyl blue formazan – MTT), 2-mercaptoethanol, 2-octanol, 4-[[4-formyl-5-hydroxyl-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid tetrasodium salt (PPADS), adenosine (ADO), adenosine 5'-diphosphate sodium salt (ADP), adenosine 5′-monophosphate sodium salt (AMP), adenosine 5'-triphosphate disodium salt (ATP), adenosine 5′-[γ-thio]triphosphate tetralithium salt (ATPγS), apyrase, bovine serum albumin (BSA), brefeldin A (BFA), bromphenol blue, carbenoxolone (CBX), Direct Red 80, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), glycerol, histamine (Hist), mefloquine (MFQ), phosphate buffered saline system (PBS), picric acid, probenecid, protease inhibitor cocktail, quinacrine dihydrochloride, reactive blue 2 (RB-2), sodium deoxycholate, sodium dodecyl sulfate (SDS), trypsin, Tween 20, type I collagenase, and cell culture reagents were purchased from Sigma-Aldrich. 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), [2-[4-[(4-Chlorophenyl)phenylmethyl]-1-piperazinyl]ethoxy]acetic acid dihydrochloride (cetirizine), 2-(propylthio)adenosine-5'-O-(β,γ-difluoro methylene) triphosphate tetrasodium salt (AR-C 66096), 2',3'-O-(2,4,6-trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) salt (TNP-ATP), 2-[(2-chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde disodium salt (MRS 2211), 2'-deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS 2179), (*S*)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-hexahydro-1*H*-1,4-diazepine dihydrochloride (H1152), ^{10}Panx and thapsigargin were obtained from Tocris Cookson Inc.. Dimethylsulphoxide (DMSO), glacial acetic acid and Triton X-100 were obtained from Merck and Bouin's solution was acquired from Panreac. BFA, H1152, MFQ, thapsigargin and U73122 were prepared in dimethylsulphoxide (DMSO). The other drugs were prepared in distilled water. DMSO (%v/v < 0.05%) did not affect the parameters under study. All stock solutions were stored as frozen aliquots at -20ºC.

ATP bioluminescence assay kit HS II was from Roche Applied Science. Bicinchoninic acid (BCA) Protein Assay Kit was from Pierce. Fluo-4 NW dye was supplied by Molecular Probes (Invitrogen).

Tissue culture plates: 96-well plates were purchased from Corning; FluoroDish plates for confocal microscopy were from World Precision Instruments;

chamber slides were from Nunc. Polyvinyl difluoride (PVDF) membranes were obtained from Millipore.

Presentation of data and statistical analysis

Data are expressed as mean ± S.E.M. from an n number of experiments/cells/individuals. Data from different individuals were evaluated one-way analysis of variance (ANOVA). Statistical differences found between control and drug-treated cultures were determined by the Bonferroni's method. *p* values < 0.05 were considered to represent significant differences.

RESULTS

Fibroblasts characterization

Cultured cells are elongated and showed a spindle-shape morphology that is characteristic of fibroblasts (94,95). Their fibroblastic nature was confirmed by immunocytochemistry. All cells exhibited a positive immunoreactivity against vimentin, the intermediate protein filament considered a reliable fibroblast-cell marker (Fig. 2.1.1A) (96). Cells also stained positive for type I collagen (Fig. 2.1.1B), which is highly produced by fibroblasts (96).

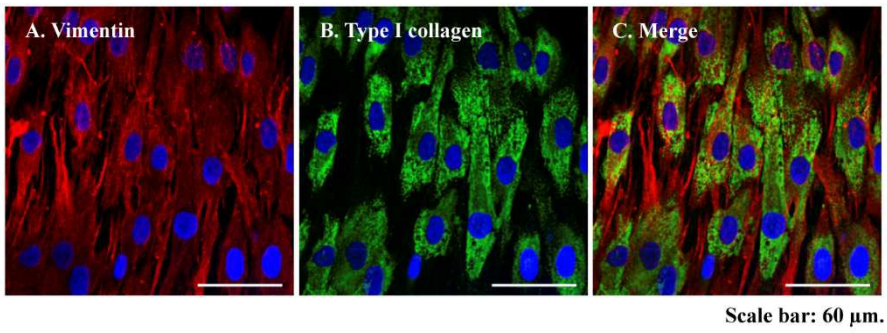

Figure 2.1.1. Immunocytochemical staining of fibroblasts isolated from the human subcutaneous tissue. Cells exhibited positive immunoreactivity against vimentin, which has been described as a reliable fibroblast marker (red, **A**). Most of the cells also stained positive to type I collagen (green, **B**), which is highly produced by activated fibroblasts. Panel **C** shows merge of vimentin and type I collagen staining. Scale bar 60 µm.

Histamine recruits Ca^{2+} from intracellular stores via the activation of H_1 receptors coupled to phospholipase C

Incubation of human subcutaneous fibroblasts with histamine (100 μM, *n* = 34) caused a fast (within seconds) rise in $[Ca^{2+}]_i$, which attained 35 ± 2% of the maximal calcium load produced by ionomycin (5 μM, 100% response) (Fig. 2.1.2B). Although the kinetics of $[Ca^{2+}]_i$ rise induced by histamine differed slightly among cells (see Fig. 2.1.2Biv), the initial fast phase was always followed by a slow decay lasting about 2 min; beyond that point, $[Ca^{2+}]_i$ levels remained fairly constant until drug washout (Fig. 2.1.2Biii).

Histamine-induced $[Ca^{2+}]_i$ oscillations were significantly ($p < 0.05$) attenuated by selective blockade of H_1 receptors with cetirizine, applied in a concentration (1 μM, *n* = 6) that on its own did not change baseline (Fig. 2.1.2C). Phospholipase C (PLC) involvement on histamine-induced $[Ca^{2+}]_i$ response was confirmed using the PLC inhibitor, U73122 (3 μM, *n* = 6) (Fig. 2.1.2D). $[Ca^{2+}]_i$ oscillations produced by histamine (100 μM, *n* = 10) were also prevented ($p < 0.05$) by the selective inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (2 μM, *n* = 4), which is known to deplete intracellular Ca^{2+} stores following a transient (< 2 min) rise of $[Ca^{2+}]_i$ levels (97) (Fig. 2.1.2E). Removal of external Ca^{2+} (Ca^{2+}-free medium plus EGTA, 100 μM, *n* = 4) depressed ($p < 0.05$) only the late component of histamine (100 μM, *n* = 13) response, keeping constant the initial fast rise (Fig. 2.1.2F).

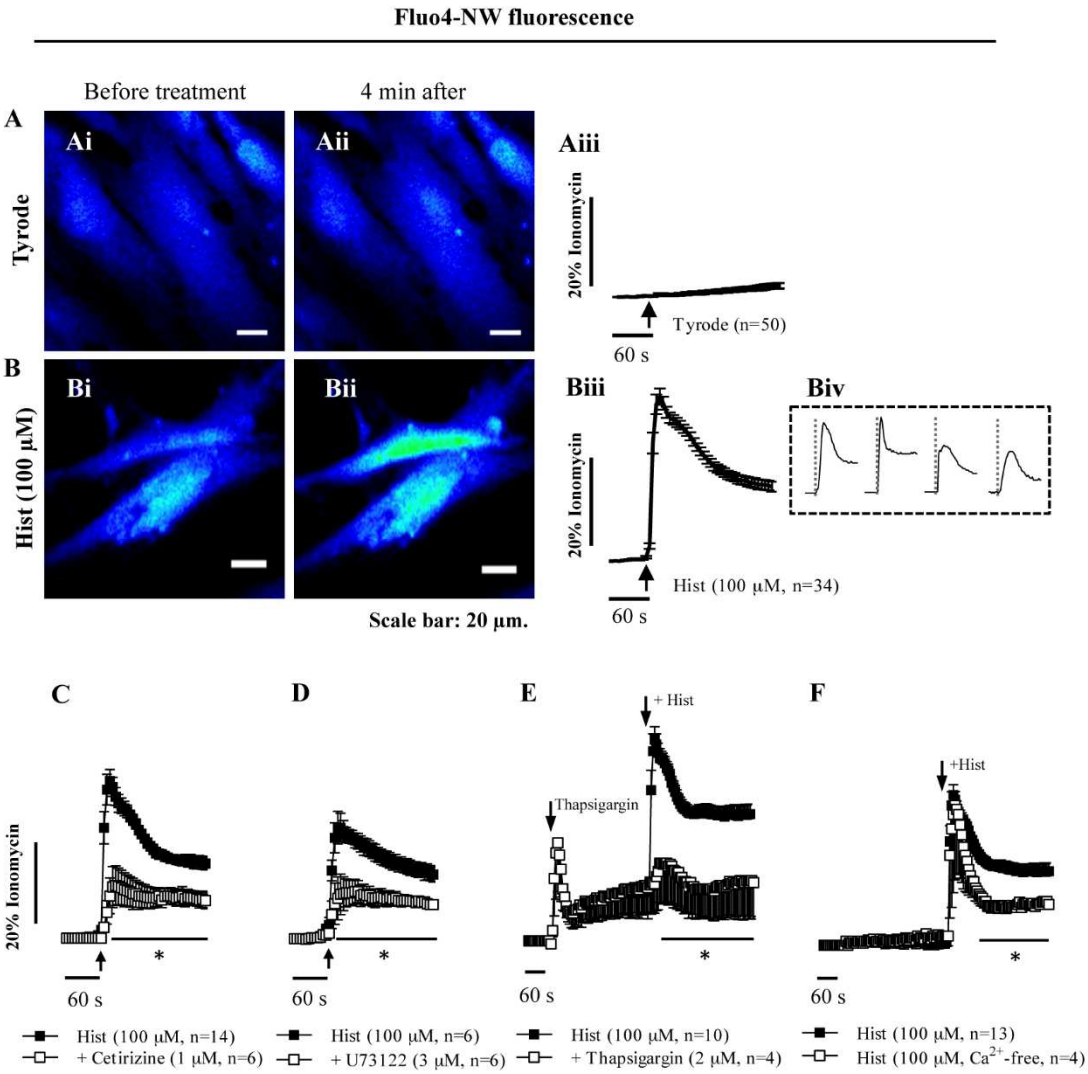

Figure 2.1.2. Histamine recruits Ca^{2+} from intracellular stores via the activation of H_1 receptors. Panels **A** and **B** represent fluorescence $[Ca^{2+}]_i$ oscillations in human subcutaneous fibroblasts in culture in the absence (**A**) and in the presence (**B**) of histamine (Hist, 100 µM). Cells were pre-incubated with the fluorescent calcium indicator, Fluo-4 NW; changes in fluorescence were detected in the time-lapse mode using a confocal microscope (**Ai**-**Aii** and **Bi**-**Bii**) or a microplate reader (**Aiii** and **Biii**) (see Experimental Procedures). Note that the kinetics of $[Ca^{2+}]_i$ signals produced by histamine differed slightly among cells (**Biv**). $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Shown are also $[Ca^{2+}]_i$ oscillations produced by Hist (100 µM) applied in the presence of the selective H_1 receptor antagonist, cetirizine (1 µM, **C**), the phospholipase C inhibitor, U73122 (3 µM, **D**), the selective endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (2 µM, **E**), and after removal of extracellular Ca^{2+} (Ca^{2+}-free medium plus EGTA, 100 µM, **F**). Black arrows indicate the time of drugs application. Each point represents pooled data from an *n* number of experiments. The vertical bars represent S.E.M. and are shown when they exceed the symbols in size. Image scale bars: 20 µm. *p < 0.05 represent significant differences from Hist (100 µM) alone.

Histamine-induced $[Ca^{2+}]_i$ accumulation is partially dependent on ATP release leading to P2 purinoceptors activation

Using the luciferin-luciferase bioluminescence assay, results show that histamine (100 μM) significantly ($p < 0.05$) increase ATP release from human subcutaneous fibroblasts as compared to the control situation where the cells were exposed to Tyrode's solution (Fig. 2.1.3A).

Extracellular ATP inactivation with apyrase (2 U/mL, $n = 9$) reduced significantly ($p < 0.05$) the late component of histamine (100 μM, $n = 26$) response, while keeping fairly conserved the magnitude of the initial $[Ca^{2+}]_i$ rise (Fig. 2.1.3Bi). A similar pattern was observed when human subcutaneous fibroblasts were pre-incubated with non-selective P2 antagonists, RB-2 (100 μM, $n = 7$) and PPADS (100 μM, $n = 6$) (Fig. 2.1.3Bii and 2.1.3Biii, respectively). Attenuation of histamine-induced $[Ca^{2+}]_i$ oscillations by PPADS and RB-2 ($p < 0.05$) contrasts with the absence of effect obtained with the preferential P2X receptor antagonist, TNP-ATP (10 μM, $n = 2$) (data not shown).

In line with these observations, exogenous application of ATP and ADP (0.1-1 mM) concentration-dependently increased $[Ca^{2+}]_i$; when applied at 1 mM concentration, ATP and ADP transiently increased $[Ca^{2+}]_i$ reaching respectively 43 ± 4% ($n = 26$) and 25 ± 8% ($n = 3$) of the maximal calcium load caused by ionomycin within 20 s from drug application. ATP-induced $[Ca^{2+}]_i$ transients were prevented ($p < 0.05$) by pre-incubation of human subcutaneous fibroblasts with PPADS (300 μM) (Fig. 2.1.3Biv).

Histamine-induced $[Ca^{2+}]_i$ mobilization depends on pannexin-1 hemichannels activity

Given that histamine-induced $[Ca^{2+}]_i$ oscillations in human subcutaneous fibroblasts depend, at least partially, on the release of ATP and subsequent P2 purinoceptors activation, we tested the action of selective inhibitors of nucleotide releasing pathways on histamine $[Ca^{2+}]_i$ responses.

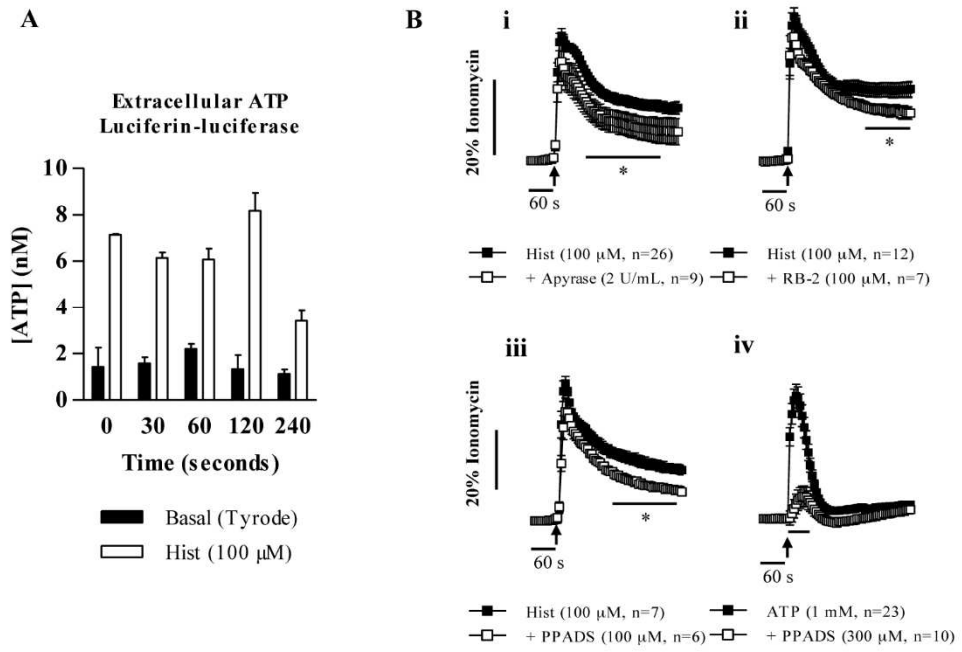

Figure 2.1.3. Histamine-induced $[Ca^{2+}]_i$ mobilization in cultured human subcutaneous fibroblasts is partially dependent on ATP release and P2 purinoceptors activation. Panel **A** illustrates the ATP (nM) content of samples from the incubation medium in the presence of histamine (Hist, 100 µM) as compared to the control condition where only Tyrode's solution was applied (luciferin-luciferase ATP bioluminescence assay). Each bar represents pooled data from 2-3 replicates of one individual. The vertical bars represent S.E.M.. Panel **B** shows the effect of histamine (Hist, 100 µM) after pretreatment of the cells with apyrase (2 U/mL, **Bi**), which catabolizes ATP and ADP into AMP, and two P2 receptor antagonists, RB-2 (100 µM, **Bii**) and PPADS (100 µM, **Biii**). For comparison purposes, we also tested the effect of ATP (1 mM, **Biv**) on $[Ca^{2+}]_i$ oscillations in the absence and in the presence of PPADS (300 µM). Cells were pre-incubated with the fluorescent calcium indicator Fluo-4 NW (see Experimental Procedures); $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Black arrows indicate the time of drugs application. No changes in baseline fluorescence were observed after application of apyrase and the two P2 purinoceptors antagonists. Each point represents pooled data from an *n* number of experiments. The vertical bars represent S.E.M. and are shown when they exceed the symbols in size. $*p < 0.05$ represent significant differences from Hist (100 µM) or ATP (1 mM) alone.

Cx43 expression is characteristic of fibroblasts from other tissue origins (78,98) and connexin-containing hemichannels are putative mediators of ATP translocation into the extracellular milieu. Inhibition of connexin hemichannels, either with mefloquine (MFQ, 3 µM, $n = 4$), which has been described to completely block Cx36- and Cx50-containing hemichannels (99,100), or with 2-octanol (1 mM, $n = 7$), which blocks Cx43, Cx46 and Cx50 hemichannels (100,101), failed to modify ($p > 0.05$) histamine-induced $[Ca^{2+}]_i$ signals (Fig. 2.1.4Ai and 2.1.4Aii, respectively). Despite the fact that 2-octanol (1 mM, $n = 7$) failed to modify $[Ca^{2+}]_i$ rises caused by histamine (100 µM), we re-evaluated the effect of MFQ increasing its concentration to 30 µM, which is high enough to

completely block Cx43-contaning hemichannels (100). Unfortunately, on its own MFQ (30 μM, n = 3) produced a yet unexplained rise ($p < 0.05$) in $[Ca^{2+}]_i$, thus precluding further studies to test the histamine response (data not shown).

Carbenoxolone (CBX, 300 μM, n = 5), a non-selective inhibitor of connexins Cx26, Cx30, Cx32, Cx43 and Cx46, which also blocks pannexin 1 (Panx1)-containing hemichannels (101), significantly ($p < 0.05$) attenuated histamine-induced $[Ca^{2+}]_i$ response (Fig. 2.1.4Aiii). The inhibitory effect of CBX (300 μM) was reproduced by the selective Panx1 mimetic inhibitory peptide, ^{10}Panx (100 μM, n = 6) (102) (Fig. 4Aiv), as well as by probenecid (150 μM, n = 4) (103), a powerful inhibitor of Panx1 hemichannels without any action on connexin-containing channels (Fig. 2.1.4Av). Compounds that affect hemichannels pore permeability, like the Rho kinase inhibitor, H1152 (3 μM, n = 6), also significantly ($p < 0.05$) decreased histamine-induced $[Ca^{2+}]_i$ rise (Fig. 2.1.4Avi).

Using immunofluorescence confocal microscopy and Western blot analysis, we confirmed that fibroblasts of the human subcutaneous tissue in culture express both Cx43 and Panx1 (Fig. 2.1.4B). In spite of this, data suggest that ATP release via Panx1 hemichannels play a more relevant role on histamine-evoked $[Ca^{2+}]_i$ mobilization under the present experimental conditions. The uptake of high molecular mass membrane-impermeable fluorescent dyes, such To-Pro3, has been used to investigate hemichannels opening (leading to the release of ATP) in viable cells by time-lapse fluorescence microscopy (104). Data presented in Fig. 2.1.5 show that perfusion of cultured fibroblasts from the human subcutaneous tissue with histamine (100 μM) caused a sustained rise of To-Pro3 dye uptake (Fig. 2.1.5Ai), which reached a maximum 30-40 s after the initial $[Ca^{2+}]_i$ peak (Fig. 2.1.5Aii). Compounds that block Panx1-containing channels, like CBX (300 μM, n = 7), or that affect hemichannels pore permeability, like H1152 (3 μM, n = 20), significantly ($p < 0.05$) decreased histamine-induced $[Ca^{2+}]_i$ rise and To-Pro3 dye uptake by human subcutaneous fibroblasts (Fig. 2.1.5B).

The involvement of nucleotide-release by exocytosis was assessed using the vesicular transport inhibitor, brefeldin A (BFA, 20 μM). No statistical significant ($p > 0.05$) differences were found in $[Ca^{2+}]_i$ oscillations produced by histamine (100 μM) in the absence and in the presence of BFA (20 μM, n = 7, Fig. 2.1.4C).

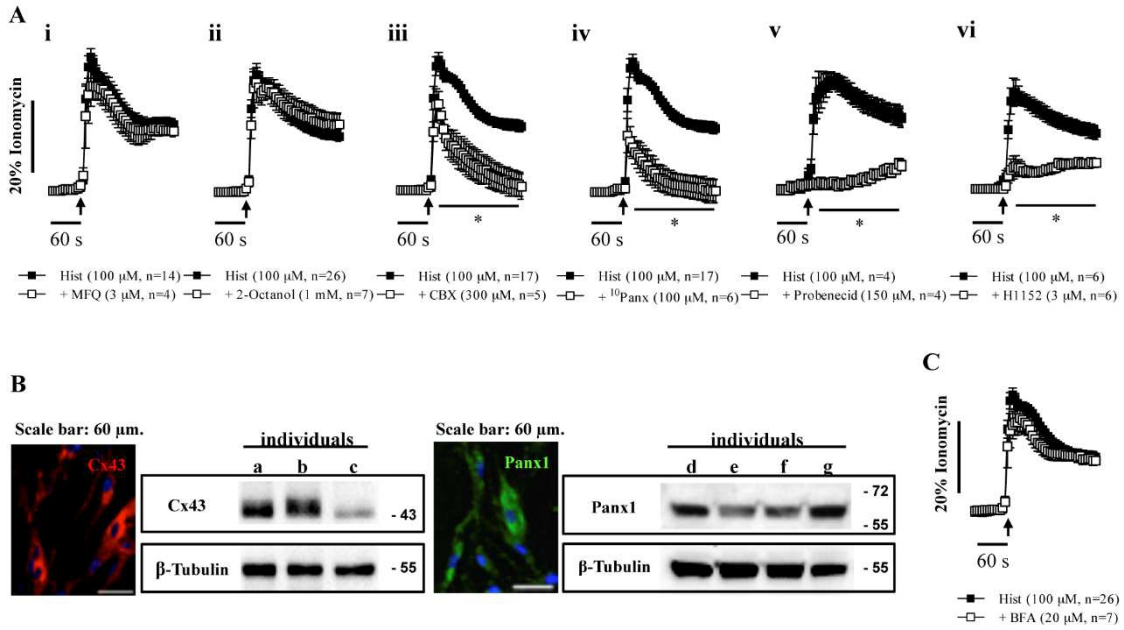

Figure 2.1.4. Histamine-induced $[Ca^{2+}]_i$ mobilization depends on pannexin-1 hemichannels activity in cultured human subcutaneous fibroblasts. The effect of histamine (Hist, 100 µM) was tested in the presence of inhibitors of connexin- and/or pannexin-containing hemichannels, namely mefloquine (MFQ, 3 µM, **Ai**), 2-octanol (1 mM, **Aii**), carbenoxolone (CBX 300 µM, **Aiii**), ^{10}Panx (100 µM, **Aiv**), probenecid (150 µM, **Av**), and H1152 (3 µM, **Avi**) as well as upon blocking vesicle exocytosis with brefeldin A (BFA, 20 µM, **C**). Cells were pre-incubated with the fluorescent calcium indicator Fluo-4 NW (see Experimental Procedures); $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Black arrows indicate the time of drugs application. None of the inhibitors significantly changed baseline fluorescence when applied alone. Each point represents pooled data from an *n* number of experiments. The vertical bars represent S.E.M. and are shown when they exceed the symbols in size. $*p < 0.05$ represent significant differences from Hist (100 µM) alone. Panel **B** shows representative confocal micrographs and blots of Cx43 and Panx1 hemichannels immunoreactivity in cultured human subcutaneous fibroblasts from several distinct individuals (a-g). β-tubulin was used as a reference protein. Image scale bars: 60 µm.

Histamine promotes the growth of human subcutaneous fibroblasts via H_1 receptors activation

Previous reports from the literature demonstrate that histamine promotes fibrosis in a number of different tissues (e.g. lung, skin) by increasing the proliferation of fibroblasts (105,106), yet the role played by histamine-induced ATP release and subsequent P2 purinoceptors activation in the growth of human subcutaneous fibroblasts has not been investigated so far. Figure 2.1.6Ai shows that cultures grown for 28 days in control conditions exhibited a gradual rise in cell viability/proliferation throughout the test period. Results concerning type I collagen production followed a similar pattern to that obtained in the MTT assay (Fig. 2.1.6Bi), indicating that under the present experimental conditions the

amount of extracellular matrix being produced depend directly on the number of viable cells in the culture.

Continuous application of histamine (10-100 µM) to the culture medium concentration-dependently increased ($p < 0.05$) human subcutaneous fibroblasts proliferation from the first week onwards. At days 21 and 28, the MTT reduction values increased ($p < 0.05$) 16 ± 4% and 27 ± 5%, respectively, in the presence of 100 µM histamine ($n = 6$), when compared to control values (Fig. 2.1.6Aii). Histamine (100 µM)-induced cells growth was significantly ($p < 0.05$) attenuated in the presence of the selective H_1 receptor antagonist, cetirizine (1 µM, $n = 3$, Fig. 2.1.6Aii). Cetirizine (1 µM) *per se* was devoid of a significant ($p > 0.05$) effect (data not shown).

The results concerning type I collagen production are shown in Figure 2.1.6Bii. Continuous treatment of the cells with histamine (10-100 µM) progressively increased ($p < 0.05$) type I collagen production from the second week onwards, in a concentration-dependent manner. A maximal increase (24 ± 4%) of type I collagen production was obtained on day 28 when the cells were incubated with 100 µM histamine ($n = 6$) (Fig. 2.1.6Bii). Selective blockade of the H_1 receptor with cetirizine (1 µM, $n = 3$) significantly ($p < 0.05$) attenuated the increase of type I collagen production caused by histamine (100 µM, Fig. 2.1.6Bii).

Because the amount of type I collagen produced depends on the number of viable cells in the culture (see above), we normalized Sirius Red absorbance values by the corresponding MTT values. This normalization eliminated the differences ($p > 0.05$) from the control situation detected on type I collagen production in the presence of histamine (100 µM) (Table 2.1.1).

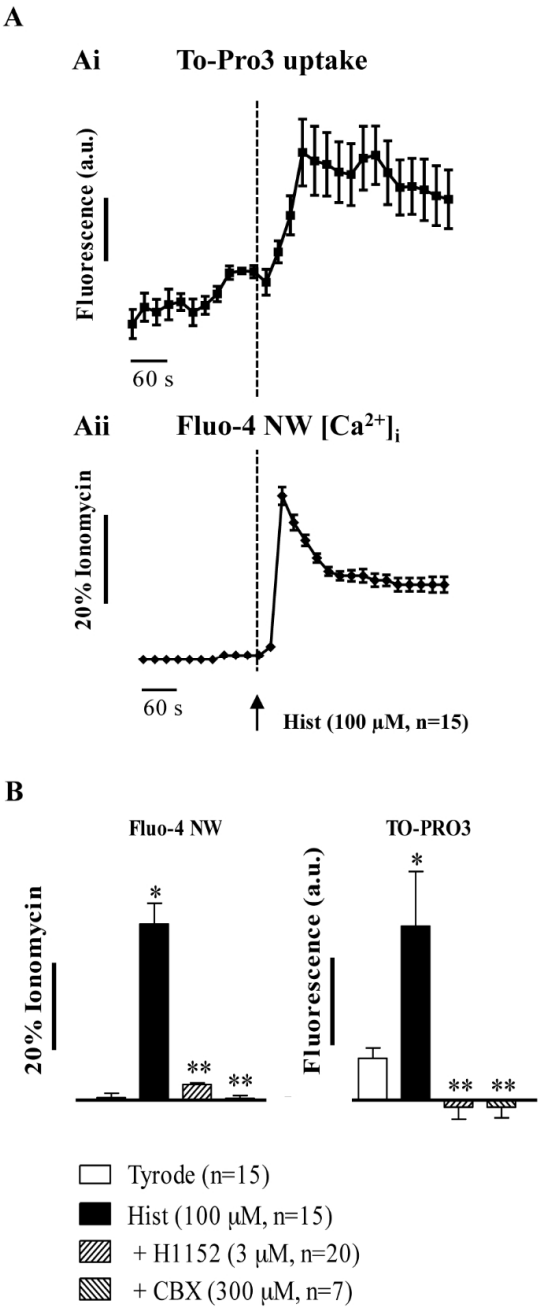

Figure 2.1.5. Histamine-induced single-cell $[Ca^{2+}]_i$ oscillations and To-Pro3 dye uptake by cultured fibroblasts of the human subcutaneous tissue evaluated by time-lapse fluorescence confocal microscopy. **A**, fluorescence variation in cells preloaded with the fluorescent calcium indicator Fluo-4NW (see "Experimental Procedures"); To-Pro3 was added to the superfusion fluid 6 min before Hist (100 µM) (dashed vertical line). Results are expressed in arbitrary units (a.u.) of fluorescence; individual $[Ca^{2+}]_i$ transients were calibrated to the corresponding maximal calcium load produced by ionomycin (5 µM; 100% response) in the same cell. **B**, bars represent the effect of Hist (100 µM) in the absence and in the presence of pannexin-1 hemichannel permeability blockers, CBX (300 µM) and H1152 (3 µM). Vertical bars represent S.E.M. from *n* experiments. $*p < 0.05$, significant differences from control values obtained in the absence of tested drugs; $**p < 0.05$, significant differences compared with the effect of Hist (100 µM) alone.

Histamine-induced human fibroblasts growth is partially dependent on $P2Y_1$ purinoceptor activation

Given that histamine promotes ATP release, we hypothesized that adenine nucleotides could be involved in the proliferative response of human fibroblasts to histamine. Continuous application of ATP (100 μM, $n = 4$) and ADP (100 μM, $n = 4$) to the culture medium increased ($p < 0.05$) cell growth (MTT assay, Fig. 2.1.6Aiii) and type I collagen production (Sirius Red assay, Fig. 2.1.6Biii) by human subcutaneous fibroblasts from the second week onwards. The pattern of nucleotides responses was similar to that obtained with histamine (100 μM) (Fig. 2.1.6Aii and 2.1.6Bii, respectively). Normalization of type I collagen production by the content of viable cells also eliminated the differences ($p > 0.05$) from the control situation (Table 1), indicating that (like histamine) adenine nucleotides have a net proliferative effect on human fibroblasts thereby increasing type I collagen content of the cultures. Interestingly, the enzymatically stable ATP analogue, ATPγS (100 μM, $n = 3$), failed to reproduce the proliferative effect of ATP (100 μM) (Fig. 2.1.6Aiii and 2.1.6Biii), thus suggesting that ATP has to be catabolized into ADP in order to promote growth of human subcutaneous fibroblasts.

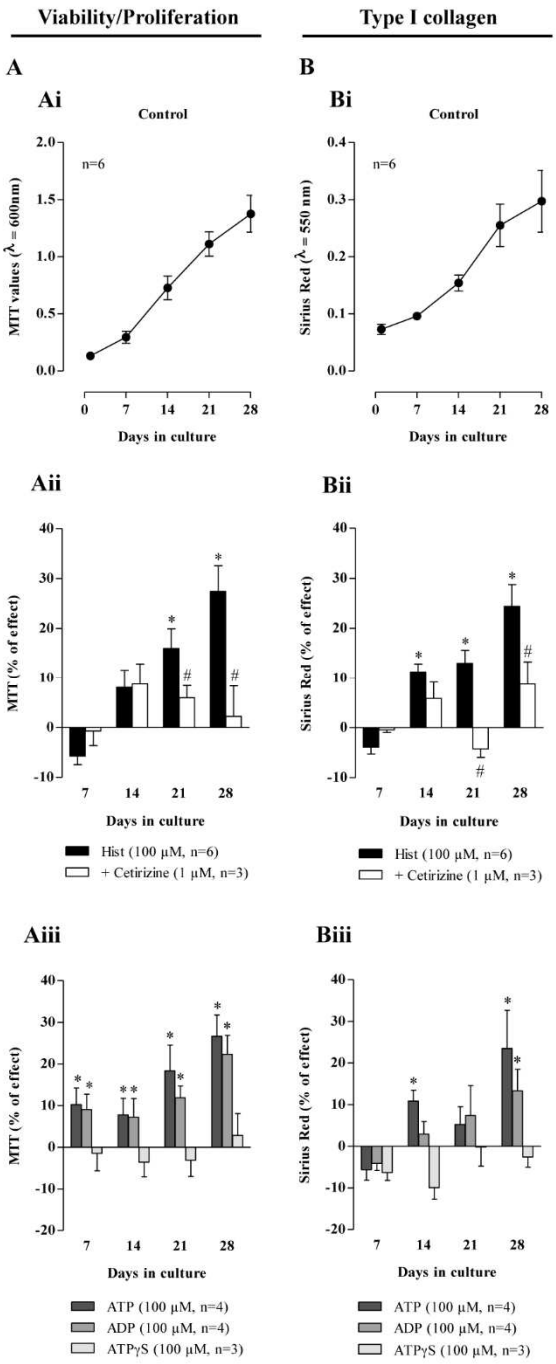

Figure 2.1.6. Proliferation/viability and type I collagen production by human subcutaneous fibroblasts grown for 28 days in culture: role of histamine and adenine nucleotides, ATP, ADP and ATPγS. Panel **Ai** represent viability/proliferation of cells measured by the MTT assay; results are expressed as absorbance determination at 600 nm per well at certain time points. Panel **Bi** represent total type I collagen production assessed by Sirius Red staining; results are expressed as absorbance determination at 550 nm per well at certain time points. Histamine (Hist, 100 μM) with or without cetirizine (selective H_1 receptor antagonist, 1 μM), ATP (100 μM), ADP (100 μM) and ATPγS (100 μM), were all applied continuously to the culture media. The ordinates represent changes in cell growth (MTT values, **Aii** and **Aiii**) and type I collagen production (Sirius Red values, **Bii** and **Biii**) as compared to controls in the absence of test drugs at the same time points (see Panels **Ai** and **Bi**). Zero represents similarity between the two values (drug *vs* control); positive and negative values represent facilitation or inhibition of either cell growth or type I collagen production relative to control data obtained at the same time points. Cetirizine (1 μM) *per se* was devoid of effect. Each column represents pooled data from 3 to 6 individuals; 4-8 replicas were performed for each individual experiment. The vertical bars represent S.E.M.. $^{*}p < 0.05$ represent significant differences from control values obtained in the absence of tested drugs; $^{\#}p < 0.05$ represent significant differences compared with the effect of Hist (100 μM) alone.

Table 2.1.1 Normalization of type I collagen production values to cell growth (MTT assay values) in human subcutaneous fibroblasts grown in culture during 28 days.

Type I collagen (λ = 550 nm)		Type I collagen (λ = 550 nm) / MTT values (λ = 600 nm)	
Control	**Hist (100 µM)**	**Control**	**Hist (100 µM)**
0.28 ± 0.02	0.35 ± 0.02 (*)	0.21 ± 0.01	0.21 ± 0.01 (n.s.)
Control	**ATP (100 µM)**	**Control**	**ATP (100 µM)**
0.26 ± 0.02	0.34 ± 0.03 (*)	0.22 ± 0.02	0.22 ± 0.01 (n.s.)
Control	**ADP (100 µM)**	**Control**	**ADP (100 µM)**
0.26 ± 0.02	0.31 ± 0.01 (*)	0.22 ± 0.02	0.21 ± 0.01 (n.s.)

Histamine (Hist, 100 µM), ATP (100 µM) and ADP (100 µM) were added continuously to the culture media of human subcutaneous fibroblasts. Because the amount of type I collagen produced depends on the number of viable cells in culture, we normalized Sirius Red absorbance values by the corresponding MTT values. Values are means ± SEM from 4 to 6 individuals; 4-8 replicas were performed for each individual experiment. *$p < 0.05$ represent significant differences from control values obtained in the absence of tested drugs (n.s. = no significance).

To investigate the contribution of ADP-sensitive P2Y purinoceptors activation to histamine-induced growth of human subcutaneous fibroblasts, we tested its effect in the presence of selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors antagonists (Fig. 2.1.7A). Selective blockade of the $P2Y_1$ receptor with MRS 2179 (0.3 µM) significantly ($p < 0.05$) attenuated the proliferative action of histamine (100 µM), but no significant differences ($p > 0.05$) were observed in the presence of AR-C 66096 (0.1 µM) and MRS 2211 (10 µM) which selectively antagonize $P2Y_{12}$ and $P2Y_{13}$ receptors, respectively (Fig. 2.1.7A). None of these antagonists modified *per se* human fibroblasts proliferation (data not shown).

The presence of ADP-sensitive P2Y purinoceptors in cultured human subcutaneous fibroblasts was confirmed by immunocytochemistry (Fig. 2.1.7B) and Western blot (Fig. 2.1.7C) analysis. Fluorescence immunoreactivity showed a cytoplasmic / membrane-staining pattern. The $P2Y_1$ receptor immunoreactivity was the most intense, followed by $P2Y_{12}$ and $P2Y_{13}$ purinoceptors, which exhibited less significant labeling intensity (Fig. 2.1.7B). As illustrated by the Western blots shown in Fig. 2.1.7Ci, the expected protein bands of human $P2Y_1$ (63 kDa), $P2Y_{12}$ (50 kDa) and $P2Y_{13}$ (41 kDa) receptors were detected in homogenates from cultured subcutaneous fibroblasts obtained from all tested individuals (a, b and c). The labeling density of ADP-sensitive P2Y receptors normalized to β-tubulin bands indicates that the $P2Y_1$ is the most expressed receptor in cultured human subcutaneous fibroblasts (Fig. 2.1.7Cii).

Pattern of the extracellular catabolism of adenine nucleotides in human subcutaneous fibroblast cultures

Figure 2.1.8 illustrates the time course of the extracellular catabolism of adenine nucleotides, ATP, ADP and AMP, in the first subculture of fibroblasts isolated from the human subcutaneous tissue. ATP (30 μM) was metabolized with a half-life time of 21.7 ± 1.4 min (n = 4 observations from two individuals). The ATP metabolites detected in the bath were ADP, adenosine (ADO) and inosine (INO), whose concentrations increased progressively with time reaching maximal values of 3.7 ± 0.7 μM, 11.1 ± 0.8 μM and 6.3 ± 0.5 μM, respectively, 30 min after ATP (30 μM) application (Fig. 2.1.8Ai). The extracellular catabolism of ADP (30 μM) was significantly (p < 0.05) slower (half-life time of 36.8 ± 3.2 min, n = 4 observations from two individuals) than that of ATP (30 μM). ADP was metabolized into ADO and INO, whose concentrations increase with time reaching maximal values of 6.6 ± 1.7 μM and 6.4 ± 2.3 μM, respectively, 30 min after ADP (30 μM) application (Fig. 2.1.8Aii). Interestingly, AMP (30 μM) was almost completely dephosphorylated into ADO and INO with a half-life time of 2.9 ± 0.7 min (n = 4 observations from two individuals), which might explain why the accumulation of AMP was almost negligible (less than 1 μM) when ATP (30 μM) and ADP (30 μM) were used as substrates (Fig. 2.1.8Aiii).

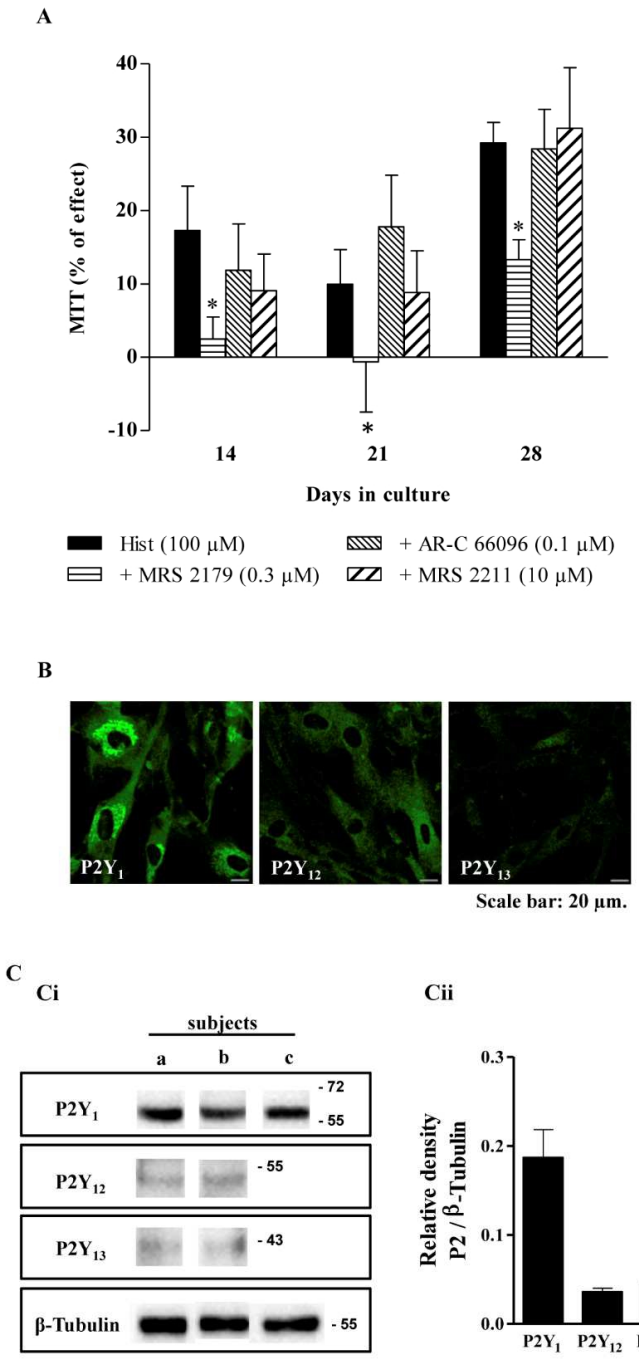

Figure 2.1.7. Histamine-induced human fibroblasts growth is partially dependent on $P2Y_1$ purinoceptor activation. Panel **A** shows the variation of cell growth caused by continuous application of histamine (Hist, 100 µM) to the culture medium in the absence and in the presence of selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor antagonists, MRS 2179 (0.3 µM), AR-C 66096 (0.1 µM) and MRS 2211 (10 µM), respectively. The ordinates represent changes in MTT values compared to control cultures grown in the absence of test drugs at the same time points (see Fig. 4). Each column represents pooled data from two individuals; 4 replicates were performed for each individual experiment. The vertical bars represent S.E.M.. $*p < 0.05$ represent significant differences compared with the effect of Hist (100 µM) alone. Panel **B** shows immunoreactivity of human subcutaneous fibroblasts against $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors at culture day 7. Images obtained under confocal microscope; representative of three independent experiments; scale bar is 20 µm. Panel **Ci** shows representative immunoblots of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors expression from three distinct individuals (a, b and c). Panel **Cii** shows the quantification of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor levels using β-tubulin as a reference protein of experiments shown in **Ci**.

Given the linearity of the semi-logarithmic representation of progress curves obtained by polynomial fitting of the catabolism of ATP ($y = -0.0134x + 1.3631$, $R^2 = 0.9334$), ADP ($y = -0.0078x + 1.4118$, $R^2 = 0.8850$) and AMP ($y = -0.0610x + 1.2594$, $R^2 = 0.9096$), the analysis of the corresponding half-life time values clearly indicates that the extracellular catabolism of ATP/ADP into AMP through E-NTPDases is the rate-limiting step to generate ADO from exogenously added adenine nucleotides, as we previously observed in human bone marrow stromal cells (49,50).

The expression of E-NTPDase family members -1, -2 and -3 and of E-5'-nucleotidase (also called CD73, EC 3.1.3.5) was assessed by immunofluorescence confocal microscopy (Fig. 2.1.8B). Human subcutaneous fibroblasts exhibited a strong immunoreactivity against E-5'-nucleotidase (Fig 2.1.8Biv), thus supporting the high AMP dephosphorylation rate detected in the enzymatic kinetic studies (Fig. 2.1.8Aiii). These cells also stained positively for E-NTPDase1 (also called CD39 or apyrase, EC 3.6.1.5) (Fig. 2.1.8Bi), as well as for E-NTPDase2 (CD39L1, EC 3.6.1.3) (Fig. 2.1.8Bii), thought for the latter the immunoreactivity was less intense. No immunoreactivity against E-NTPDase3 (CD39L3 or HB6) was detected in these cells (Fig. 2.1.8Biii).

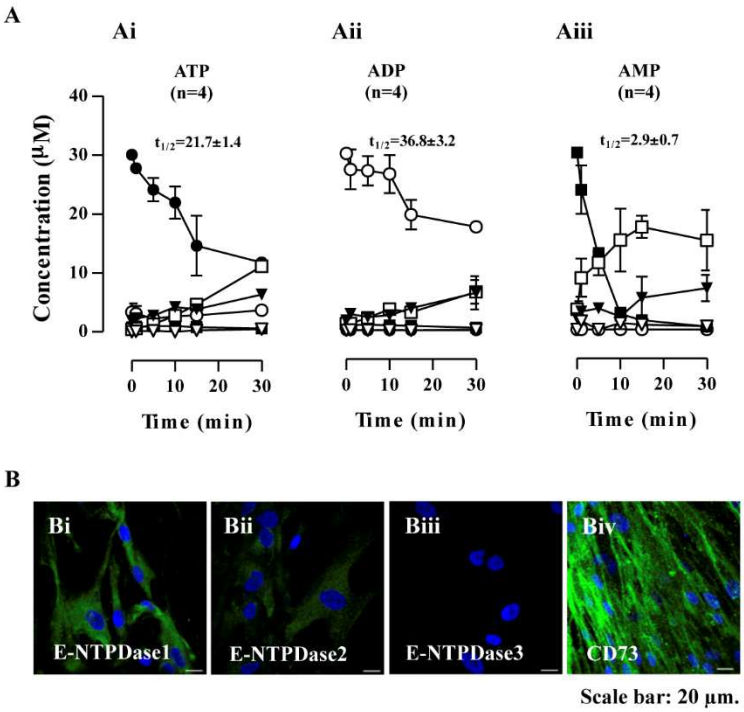

Figure 2.1.8. Time course of the extracellular catabolism of adenine nucleotides and immunocytochemical detection of E-NTPDases in human subcutaneous fibroblast cultures at day 11. ATP (**Ai**), ADP (**Aii**) and AMP (**Aiii**) were added at a concentration of 30 μM to the culture medium at time zero. Samples (75 μL) were collected from each well at indicated times in the abscissa. Each collected sample was analyzed by HPLC to separate and quantify ATP (filled circles), ADP (open circles), AMP (filled squares), adenosine (ADO, open squares), inosine (INO, filled triangles) or hypoxanthine (HX, open triangles). Each point represents pooled data from two individuals; 2 replicas were performed in each individual experiment. Vertical bars represent S.E.M. and are shown when they exceed the symbols in size. The calculated half-life time ($t_{1/2}$, min) for each initial substrate is shown for comparison. Panel **B** shows immunoreactivity of human subcutaneous fibroblasts against E-NTPDase1, E-NTPDase2, E-NTPDase3 and E-5'-nucleotidase (CD73). Cells grown in eight-well chamber slides were processed for immunocytochemistry in parallel and were visualized keeping unaltered the settings of the confocal microscope throughout the procedure. Scale bar is 20 μm.

DISCUSSION

Histamine belongs to a group of endogenous chemical substances that are released upon cell damage or during inflammatory insults (107-109). The role of histamine in nociception is not clear but it appears to cooperate with the effect of other endogenous mediators (23). It has been shown that histamine, together with substance P, is capable of inducing ATP release from smooth muscle cells of the guinea-pig vas deferens (83). To the best of our knowledge, this is the first study demonstrating that human fibroblasts isolated from subcutaneous connective tissue respond to histamine by releasing ATP into the extracellular media through the activation of H_1 receptors.

Results also showed that histamine triggers intracellular Ca^{2+} mobilization via the activation of G_q protein-coupled H_1 receptors, given that inhibition of PLC with U73122 and depletion of intracellular Ca^{2+}-stores caused by thapsigargin attenuated its response. Moreover, extracellular Ca^{2+} might also play some role to sustain the late component of histamine-induced $[Ca^{2+}]_i$ rise, since its removal from the incubation medium attenuated significantly histamine response. A similar response to histamine has been reported in epithelial cells (110). Our findings could be explained by a possible involvement of store-operated calcium channels in order to compensate the fall of the $[Ca^{2+}]_i$ content (111) and/or through a concurrent activation of other membrane receptors, namely P2 purinoceptors. Attenuation of histamine-induced $[Ca^{2+}]_i$ response by apyrase suggests the cooperation with a nucleotide mediator, such as ATP and/or ADP. Since blockade of P2 purinoceptors mimicked the attenuating effect of apyrase, our data provide the first evidence that histamine-induced $[Ca^{2+}]_i$ accumulation by fibroblasts of human subcutaneous tissue is partially mediated by the release of ATP and subsequent activation of P2 purinoceptors. Recently, it was demonstrated that stretch-induced ATP release in rat subcutaneous fibroblasts influence cytoskeletal remodeling of the cells in part by the subsequent activation of P2 purinoceptors (70).

Depending on the cell type, there are multiple nucleotide-releasing pathways, which represent a critical component for the initiation of the purinergic signaling cascade (36). Considering that Ca^{2+} signaling induced by histamine seems to partially involve P2 purinoceptors activation subsequent to the release of ATP from fibroblasts of the human subcutaneous tissue, we assessed the contribution of several nucleotide-releasing mechanisms by measuring $[Ca^{2+}]_i$ oscillations. Histamine-induced $[Ca^{2+}]_i$ oscillations were not affected upon inhibition of the vesicular transport with BFA, thus suggesting that exocytosis might not represent an important pathway for releasing ATP in these cells. Further studies designed to investigate unloading of vesicular ATP stores stained with quinacrine are required to exclude this possibility in human fibroblasts (112). Here, we proved that human subcutaneous fibroblasts express Cx43 and Panx1 hemichannels. Although both channels have been committed to translocation of nucleotides to the extracellular milieu in numerous cell types, functional data using non-selective connexin inhibitors, such as 2-octanol and MFQ, failed to modify histamine-induced $[Ca^{2+}]_i$ oscillations in fibroblasts of the human subcutaneous tissue. Conversely, blockade of Panx1-containing

hemichannels with CBX (101), ^{10}Panx (102) and probenecid (103) significantly attenuated the histamine-induced $[Ca^{2+}]_i$ response. Given that probenecid is a powerful Panx1 inhibitor with no effect on connexin channels, our findings suggest that Panx1-containing hemichannels might have a predominant role on histamine-induced $[Ca^{2+}]_i$ responses operated by ATP released from human subcutaneous fibroblasts. The reason for the disparity between protein expression and function may be because Cx43, unlike Panx1, hemichannels close at normal milimolar Ca^{2+} (*e.g.* 1.8 mM $CaCl_2$ in Tyrode's solution) and open upon external Ca^{2+} depletion (113).

The involvement of ATP releasing hemichannels was further confirmed by time-lapse fluorescence microscopy focusing on the uptake of To-Pro3 by cultured human fibroblasts. Following the initial $[Ca^{2+}]_i$ peak, histamine caused a sustained increase in To-Pro3 dye uptake by the cells, which paralleled the second component of $[Ca^{2+}]_i$ rise. Sustained effects of histamine on both $[Ca^{2+}]_i$ and To-Pro3 dye uptake were significantly attenuated upon blocking pannexin-1 hemichannels permeability with CBX and H1152, thus implicating the opening of large membrane pores that facilitate translocation of high molecular mass molecules (like ATP) between intra- and extracellular compartments.

The molecular mechanism(s) by which histamine releases ATP through the opening of Panx1 hemichannels in human subcutaneous fibroblasts deserves further investigations. Nevertheless, disregarding whether channel mediated efflux or vesicle exocytosis comprises the predominant ATP release mechanism, most studies have identified elevation of cytosolic Ca^{2+} as an important regulator of ATP release in different cell models. Generation of inositol trisphosphate by phospholipase C may be the process underlying elevation of cytosolic Ca^{2+} (114,115). Histamine H_1 receptor, like other G_q-coupled receptors, may additionally stimulate Rho-GTPase (via $G_{12/13}$) acting to strongly potentiate the Ca^{2+}-activated ATP release pathway (116,117). Rho activation and Ca^{2+} mobilization must be temporally coordinated to promote ATP release. Interestingly, Seminario-Vidal and col. (2009) (117) demonstrated that Ca^{2+}- and RhoA/Rho kinase-dependent ATP release from thrombin-stimulated A549 lung epithelial cells occurs via connexin or pannexin hemichannels, but this pathway might not be competent for ATP release in human astrocytoma cells (116). Given the actions exerted by Rho/Rho kinase on scaffold proteins and cytoskeleton components (*e.g.* regulating myosin-light chain phosphorylation and actin polymerization), authors speculate that Rho-promoted membrane-cytoskeleton rearrangements

facilitate the insertion of hemichannel subunits within the plasma membrane. Blockade of one of these multiple mechanisms might explain the attenuating effect of the Rho kinase inhibitor, H1152, on histamine-induced $[Ca^{2+}]_i$ rise observed in this study. These findings suggest that RhoA/Rho kinase activation is a step upstream of Panx1-mediated ATP release triggered by histamine in human subcutaneous fibroblasts.

Our data also demonstrate that fibroblasts of human subcutaneous connective tissue respond to histamine by increasing cell growth and subsequent type I collagen production through the activation of H_1 receptors. Normalization of type I collagen production by the number of viable cells indicates that histamine exerts a preferential effect on cell proliferation rather than in extracellular matrix remodeling. In lungs and skin, histamine exerts a pro-fibrotic action by increasing cells growth (105,106) and collagen deposition (106,118) via H_2 receptors activation. Despite the close anatomical proximity between skin and subcutaneous connective tissue, functional differences between fibroblasts from different tissue origins have been described (119,120). Thus, differences between our findings and previous reports regarding the receptor subtype involved in the action of histamine may be explained by differences in tissue and species origin. On the other hand, distinct methodological approaches have been used. Previous studies (105,106,118) exposed cells to histamine from several hours to a few days (7 days), whereas we monitored histamine-induced effects in cells kept in culture for 28 days. Moreover, in contrast to previous studies, we found significant differences in cells growth and type I collagen production induced by histamine (10-100 μM) exposure from the second week onwards. Actually, we did not monitor acute (for hours to few days) changes operated by histamine in our cultures. These findings strengthen the idea that, although fibroblasts are ubiquitous mesenchymal cells, they might function differently depending on their location.

Given that histamine triggered the release of ATP from human subcutaneous fibroblasts in culture, we postulated that adenine nucleotides could be involved in the proliferative response of histamine in these cells. Continuous application of ATP and ADP to the culture medium mimicked the proliferative effect of histamine, indicating that adenine nucleotides promote human fibroblasts growth and subsequent type I collagen production. Surprisingly, no such effect was found with the enzymatically stable ATP analogue, ATPγS, Thus, it seems that cells exposed to histamine release ATP into the extracellular milieu,

which, upon conversion into ADP, indirectly promotes growth of human subcutaneous fibroblasts. In fact, selective blockade of ADP-sensitive $P2Y_1$ receptors attenuated histamine-induced cells proliferation, but no changes were detected in the presence of the selective $P2Y_{12}$ and $P2Y_{13}$ antagonists. The presence of $P2Y_1$ receptors in fibroblasts of the human subcutaneous tissue was confirmed by immunofluorescence confocal microscopy and Western blot analysis. Data show that immunoreactivity against the $P2Y_1$ receptor was much more intense as compared to that of $P2Y_{12}$ and $P2Y_{13}$ receptors. Functional data and immunolabeling experiments are consistent with ADP-sensitive $P2Y_1$ being the receptor subtype mediating the purinergic loop leading to cell growth in response to histamine. This outcome is particularly interesting considering that the $P2Y_1$ receptor, along with $P2Y_2$ and P2X7 subtypes, have been implicated in chronic inflammation (44). These authors used disks made of non-cytotoxic degradable dermal sheep collagen implanted subcutaneously in rats to monitor the foreign body inflammatory reaction during 21 days. They found that $P2Y_1$, $P2Y_2$ and P2X7 receptors were upregulated, in parallel to vascular activation and increased number of macrophages and giant cells. Although fibroblasts were shown to encapsulate the disks and to promote deposition of extracellular matrix components (44), these cells were not further evaluated. Given that inflammatory reactions involve the recruitment of mast cells, which release huge amounts of histamine to the extracellular milieu, we speculate that activated fibroblasts would proliferate, thus contributing to the global increase in $P2Y_1$ receptors expression.

The activity of ecto-nucleotidases is critical to define the inactivation rate of adenine nucleotides within close proximity of the release sites in tissue microenvironments. In this study we analyzed the role of three members of the ecto-nucleoside triphosphate diphospho- hydrolase (E-NTPDase), namely E-NTPDase1, E-NTPDase2, and E-NTPDase3 (121). These enzymes have distinct biochemical properties. E-NTPDase1 hydrolyzes ATP and ADP equally well, E-NTPDase2 preferentially hydrolyzes triphosphonucleosides, and E-NTPDase3 has an intermediate hydrolysis profile (39). The hydrolysis of tri- and diphosphonucleosides by E-NTPDases yields AMP as the final product which can be fully dephosphorylated to adenosine by E-5'-nucleotidase/CD73 (122). Our results showed that human subcutaneous fibroblasts exhibit a strong immunoreactivity against E-5'-nucleotidase/CD73, which is in keeping with a faster conversion of AMP into adenosine. A high E-5'-nucleotidase/CD73 activity

might also explain why the concentration of AMP was kept at low level when ATP and/or ADP were used as substrates (123).

Luttikhuizen and col. (2004) (44) reported the overexpression of subcutaneous E-NTPDase1 in a model of chronic inflammation. Interestingly, we showed here that human subcutaneous fibroblasts express E-NTPDase1 immunoreactivity under resting conditions. This member of the NTPDase family seems to be the most expressed ATP and/or ADP metabolizing enzyme in these cells, as predicted from lesser immunofluorescence signals obtained for other E-NTPDases. These cells exhibit weak immunoreactivity against E-NTPDase2, and no signal was detected for E-NTPDase3, suggesting that E-NTPDase2 may participate together with E-NTPDase1 in the extracellular catabolism of adenine nucleotides in fibroblasts of the human subcutaneous tissue. E-NTPDase1, E-NTPDase2, and E-NTPDase3 hydrolyze extracellular tri- and diphosphonucleotides with ATP:ADP ratios of ~1-2:1, ~10-40:1, and ~3-4:1, respectively (39). The kinetic analysis of the extracellular catabolism of ATP and ADP in human subcutaneous fibroblast cultures indicates that adenine nucleotides are metabolized with a ratio of approximately 1.5(ATP):1(ADP), which is compatible with E-NTPDase1 being the most effective isoform in these cultures. In line with this hypothesis, one would expect that the formation of adenosine should be prevented if NTPDase2 acted alone or it could be delayed if NTPDases3 was the main enzyme involved in the catabolism of adenine nucleotides, given that it is well known that E-5'-nucleotidase/CD73 is inhibited by ADP (39,123). Our results proved exactly the opposite showing that AMP was rapidly dephosphorylated into adenosine independently of the nucleotide substrate used. On the other hand, involvement of E-NTPDase1 is expected to terminate the effects exerted by either ATP or ADP sensitive receptors. Nevertheless, data indicate that responses of human subcutaneous fibroblasts to histamine depend on the activation of ADP-sensitive $P2Y_1$ receptors. This situation can occur, because E-NTPDase2 concurs with E-NTPDase1 to release enough ADP required to activate $P2Y_1$ receptors in human subcutaneous fibroblasts, as the former enzyme preferentially hydrolyzes triphosphonucleosides.

In conclusion, our findings showed for the first time that histamine promotes the release of ATP from human subcutaneous fibroblasts via Panx1 hemichannels leading to $[Ca^{2+}]_i$ mobilization from internal stores and cell growth through the cooperation of H_1 and P2Y receptors (most probably of the $P2Y_1$ subtype) activation. Targeting the pathways leading to nucleotides release and

the purinergic cascade, consisting in metabolizing E-NTPDases and P2 purinoceptors activation, may be useful in designing novel therapies towards the modulation of cell signals between fibroblasts, nociceptors and inflammatory cells, which underlie the pathogenesis of painful musculoskeletal diseases with widespread involvement of the subcutaneous connective tissue, such as fibromyalgia.

PAPER 2

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Bradykinin-induced Ca^{2+} signaling in human subcutaneous fibroblasts involves ATP release via hemichannels leading to $P2Y_{12}$ receptors activation

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ABSTRACT

Background – Chronic musculoskeletal pain involves connective tissue remodeling triggered by inflammatory mediators, such as bradykinin. Fibroblast cells signaling involve changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$). ATP has been related to connective tissue mechanotransduction, remodeling and chronic inflammatory pain, via P2 purinoceptors activation. Here, we investigated the involvement of ATP in bradykinin-induced Ca^{2+} signals in human subcutaneous fibroblasts.
Results – Bradykinin, via B_2 receptors, caused an abrupt rise in $[Ca^{2+}]_i$ to a peak that declined to a plateau, which concentration remained constant until washout. The plateau phase was absent in Ca^{2+}-free medium; $[Ca^{2+}]_i$ signal was substantially reduced after depleting intracellular Ca^{2+} stores with thapsigargin. Extracellular ATP inactivation with apyrase decreased the $[Ca^{2+}]_i$ plateau. Human subcutaneous fibroblasts respond to bradykinin by releasing ATP via connexin and pannexin hemichannels, since blockade of connexins, with 2-octanol or carbenoxolone, and pannexin-1, with ^{10}Panx, attenuated bradykinin-induced $[Ca^{2+}]_i$ plateau, whereas inhibitors of vesicular exocytosis, such as brefeldin A and bafilomycin A1, were inactive. The kinetics of extracellular ATP catabolism favors ADP accumulation in human fibroblast cultures. Inhibition of ectonucleotidase activity and, thus, ADP formation from released ATP with POM-1 or by Mg^{2+} removal from media reduced bradykinin-induced $[Ca^{2+}]_i$ plateau. Selective blockade of the ADP-sensitive $P2Y_{12}$ receptor with AR-C66096 attenuated bradykinin $[Ca^{2+}]_i$ plateau, whereas the $P2Y_1$ and $P2Y_{13}$ receptor antagonists, respectively MRS 2179 and MRS 2211, were inactive. Human fibroblasts exhibited immunoreactivity against connexin-43, pannexin-1 and $P2Y_{12}$ receptor.
Conclusions – Bradykinin induces ATP release from human subcutaneous fibroblasts via connexin and pannexin-1-containing hemichannels leading to $[Ca^{2+}]_i$ mobilization through the cooperation of B_2 and $P2Y_{12}$ receptors.

Keywords – ADP, ATP release, bradykinin, Cx43, hemichannels, human subcutaneous fibroblasts, Panx1, P2 purinoceptors

INTRODUCTION

Despite its overwhelming size throughout the body, the connective tissue has been generally overlooked or misunderstood. It has been considered as relatively superfluous apart from its supporting role amongst more specialized tissues (1). It has long been known that scar tissue is a common cause of chronic musculoskeletal pain. Evidences have been produced suggesting that connective tissue may become thicker and less compliant in patients with chronic pain, possible as a result of chronic inflammation and fibrosis (12-14,16). Therefore, the normal response to mechanical stretch may be dampened by disturbance of the viscoelastic properties of the subcutaneous connective tissue as a consequence of fibroblast remodeling promoted by inflammatory mediators, like neurotrophins, cytokines, peptides, protons, free radicals, histamine, bradykinin, serotonin, and prostanoids (82). Since the subcutaneous connective tissue is richly innervated by sensory nerve endings, inputs arising from affected connective tissue may alter pain perception.

Bradykinin is one of the most potent algogenic compounds that is synthesized from inactive precursors, the kininogens, following tissue injury (124) and by contracting skeletal muscles (reviewed in (125)). Bradykinin has been strongly implicated in tissue inflammation (124) and it is also known to be mitogenic in fibroblasts from the human foreskin and lung (126,127). Bradykinin preferentially induces its physiological effects by binding to the B_2 receptor subtype. In intact cells, bradykinin was shown to induce the activation of phospholipases A_2, C and D; the release of prostaglandins, the accumulation of cyclic AMP and of cyclic GMP, and the mobilization of Ca^{2+} were demonstrated (128). Bradykinin causes a rapid (within 30 s) translocation of protein kinase C isoforms of all groups (classical Ca^{2+}-dependent isoform α, new Ca^{2+}-independent isoform ε, and atypical isoform ζ) from the cytosol to the plasma membrane (128). Bradykinin-induced translocation of protein kinase C to the plasma membrane may favor enzyme coupling to coexistent extracellular signaling molecules under pathological conditions, thus significantly potentiating their effects.

The way bradykinin is involved in pain perception might involve direct excitation of primary nociceptive afferents and/or the indirect reduction of nociceptors threshold by favoring the release of excitatory signaling mediators (22). It has been showed that acute bradykinin exposure potentiates algogenic P2X3 purinoceptor-mediated calcium responses from neurons, followed by their down-regulation upon chronic (24 h) exposure. On the other hand, P2Y receptors

responses in satellite neuroglia may be upregulated, suggesting a complex interplay between bradykinin and P2 purinoceptors activation in pain pathophysiology (129). Previous studies demonstrated that bradykinin elicits the release of ATP from various cell types, including smooth muscle fibers, epithelial cells and cardiac endothelial cells from guinea pigs (83), urothelial cells from both human and rats (130,131), and several immortalized cell lines (*e.g.* MDCK, COS-7, HEK-293) (reviewed in (132)). The mechanism of ATP release induced by bradykinin is, however, poorly understood, particularly in human tissues. Nucleotides-releasing pathways in intact cells include [1] electrodiffusional translocation via connexin- and pannexin-containing hemichannels and voltage-dependent anion channels, [2] facilitated diffusion by nucleotide-specific ATP-binding cassette (ABC) transporters, and [3] vesicle exocytosis (36). In parallel to bradykinin, huge amounts of extracellular ATP may leak from damaged cells during mild tissue injury. Once released, ATP may act as an autocrine or paracrine mediator in neighboring cells via ionotropic P2X and metabotropic P2Y purinoceptors activation. ATP signaling may, however, be limited by membrane-bound ectonucleotidases, which sequentially catabolize nucleoside 5'-triphosphates to their respective 5'-di- and monophosphates and adenosine (36). As a consequence, appearance of ATP and active metabolites, like ADP and adenosine, in the extracellular fluid form concentration gradients enabling differential targeting of subtype-specific purinoceptors and, thus, cell communication and signaling.

Thus, taking into consideration that [1] changes in the regulation of connective tissue ATP signaling may be important in the pathogenesis of chronic inflammatory pain (44) and that [2] algogenic inflammatory mediators, such as bradykinin, may sensitize cells to autocrine and paracrine signals operated by extracellular adenine nucleotides (77), we investigated the involvement of ATP in bradykinin-induced Ca^{2+} signals in human subcutaneous fibroblasts. Understanding the mechanisms underlying purinergic cell signaling and its interplay with inflammatory mediators in the human subcutaneous connective tissue may highlight new strategies for the treatment of chronic musculoskeletal painful diseases (*e.g.* drug-resistant fibromyalgia).

EXPERIMENTAL PROCEDURES

Cell cultures

Human fibroblasts were isolated from the subcutaneous tissue of organ donors (51 ± 6 years old (mean ± S.E.M.), *n* = 13) with no clinical history of connective tissue disorders. The protocol was approved by the Ethics Committee of Centro Hospitalar do Porto (University Hospital) and of Instituto de Ciências Biomédicas de Abel Salazar (Medical School) of University of Porto (approval No. 11-CES-05). The investigation conforms to the principles outlined in the Declaration of Helsinki. Subcutaneous tissues were maintained at 4-6ºC in M-400 transplantation solution (4.190 g/100 mL mannitol, 0.205 g/100 mL KH_2PO_4, 0.970 g/100 mL $K_2HPO_4 \cdot 3H_2O$, 0.112 g/100 mL KCl, and 0.084 g/100 mL $NaHCO_3$, pH 7.4) until used, which was between 2 and 16 hours after being harvested (84). Cells were then obtained by the explant technique and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 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 3-4 weeks, until near confluence, when adherent cells were enzymatically released with 0.04% trypsin-EDTA solution plus 0.025% type I collagenase in phosphate-buffered saline (PBS), at pH 7.4 during 15-20 minutes. The resultant cell suspension was cultured and maintained in the same conditions mentioned above. All the experiments were performed in the first subculture.

Intracellular ATP imaging by quinacrine staining

Quinacrine is a weak-base acridine derivative that binds ATP with high affinity and specificity. When excited by light at 476 nm it emits fluorescence in the 500-540 nm range and it has been widely used to visualize ATP-containing sub-cellular compartments in living cells (133,134). Human fibroblasts were seeded on 35 mm glass bottom dishes (FluoroDish®, World Precision Instruments), at a density of $2x10^4$ cells/mL, and maintained in culture for 5-15 days. To visualize ATP-filled vesicles, dishes were washed twice with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 11.2 mM glucose, pH 7.4) before incubation with 30 μM quinacrine for 1 h (134); cells were washed again twice and mounted onto a thermostatic (32ºC) perfusion chamber at the stage of an inverted laser-

scanning confocal microscope (Olympus FluoView™ FV1000) equipped with a 40x magnification oil immersion objective lens (UPLAPO 40x OI; NA: 1.00). The chamber was continuously superfused (1 mL/min) with gassed (95% O_2 + 5% CO_2, pH 7.4) Tyrode's solution and the cells were immediately observed. Changes in quinacrine fluorescence were detected in the time-lapse mode with FluoView Advanced Software (Olympus). Quinacrine was excited with a 488 nm Multi-line Ar laser and the emitted fluorescence was detected at 500-550 nm, using the scanner of the confocal microscope. Drugs were delivered using a multichannel perfusion system (ValveLink 8.2, Digitimer). Time-lapse sequences were recorded at scanning rates of 3 seconds per image, every 5 seconds, for about 30 minutes, digitized, and processed off-line by the computer. Regions of interest were defined as bright areas with as little background as possible.

Extracellular ATP quantification by bioluminescence

Extracellular ATP was detected with the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science) using a Turner BioSystems (TD 20/20^n) luminometer that accommodates 35-mm culture dishes, as described elsewhere (135). Briefly, cells were seeded on 35 mm glass bottom dishes, at a density of $2x10^4$ cells/mL, for 5-15 days. At the beginning of the experiment, cells were washed twice with Tyrode's solution at room temperature, before placing the dish in the luminometer reaction chamber. Bradykinin (30 μM) was added manually to the incubation fluid when bioluminescence baseline values reached stability after application of the luciferin/luciferase reagent. Experiments were performed at room temperature. ATP-dependent changes in extracellular luciferase activity were measured as relative luminescence unit (RLU) values integrated over 10 seconds photon counting periods.

Measurement of intracellular Ca^{2+}

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured with the calcium sensitive dye Fluo-4 NW (Fluo-4 NW calcium assay kit, Molecular Probes, Invitrogen) in a multi detection microplate reader (Synergy™ HT Multi-Mode Microplate Reader, BioTek Instruments) (85). In some of the experiments, single-cell $[Ca^{2+}]_i$ imaging was obtained using a laser scanning confocal microscope (49). Briefly, human fibroblasts were seeded in flat bottom 96 well plates (Costar®, Corning® Inc.) at a density of $3x10^4$ cells/mL for the experiments

using the microplate reader, and into 35 mm glass bottom dishes at a density of $2x10^4$ cells/mL for single-cell $[Ca^{2+}]_i$ imaging. Cells were cultured for 5-15 days in supplemented DMEM as described before. On the day of the experiment, cells were washed twice with Tyrode's solution and incubated at 37ºC for 45 minutes with the cell-permeant fluorescent Ca^{2+} indicator, Fluo-4 NW (2.5 μM), in 1X HBSS, 20 mM HEPES and 2.5 mM probenecid. After removal of the fluorophore loading solution, cells were washed again twice and 150/300 μL of Tyrode's solution was added per culture well/dish, respectively. The 96-well cell plates were then loaded into the microplate reader. Reader control was performed using the BioTek's Gen5™ Data Analysis Software. For the recordings, temperature was maintained at 32ºC and readings were made with 5 seconds of interval, during approximately 30 minutes, using a tungsten halogen lamp. Fluorescence was excited at 485/20 nm and emission was measured at 528/20 nm. For the single-cell imaging, culture dishes were mounted on a thermostatic (32ºC) perfusion chamber at the stage of an inverted laser-scanning confocal microscope equipped with a 20x magnification objective lens (LUCPLFLN 20x PH; NA: 0.45). The chamber was continuously superfused (1 mL/min) with gassed (95% O_2, 5% CO_2, pH 7.4) Tyrode's solution and drugs were delivered using a multichannel valve controlled perfusion system. Changes in fluorescence of the Fluo-4 NW dye were detected in a time-lapse mode. Fluo-4 NW was excited with a 488 nm Multi-line Ar laser and the emitted fluorescence was detected at 510-560 nm, using the scanner of the confocal microscope. Time-lapse sequences were recorded at scanning rates with 20 seconds of interval for approximately 30 minutes, digitized, and processed off-line by the computer. Regions of interest were defined as bright areas with as little background as possible. For both methods, calcium measurements were calibrated to the maximal calcium load produced by ionomycin (5 μM, 100% response). In some experiments, we used rat subcutaneous fibroblasts obtained using the method described before to monitor single-cell $[Ca^{2+}]_i$ oscillations (see above) in parallel with the incorporation of FM4-64 (Molecular Probes, Invitrogen), which is a membrane-selective fluorescent dye used to evaluate vesicle endocytosis and exocytosis in living cells (136,137). FM4-64 was excited with a 488 nm Multi-line Ar laser and fluorescence emission was detected at 665-765 nm, using the scanner of the confocal microscope. Drug application and time-lapse sequences were performed as above.

Enzymatic kinetic experiments and HPLC analysis

The kinetics of ATP, ADP and AMP hydrolysis in human fibroblast cell cultures was evaluated at day 11, at 37ºC. After a 30 minutes equilibration period, cells were incubated with 3 μM ATP, ADP or AMP, which were added to the culture medium at zero time. Samples (75 μL) were collected from each well at different times up to 30 minutes for high-performance liquid chromatography (HPLC, LaChrome Elite, Merck) analysis of the variation of substrate disappearance and product formation (49,84). Concentrations of the substrate and products were plotted as a function of time (progress curves). The following parameters were analyzed for each progress curve: half-life time ($t_{1/2}$) of the initial substrate, time of appearance of the different concentrations of the products, concentration of the substrate or any product remaining at the end of the experiment.

Immunocytochemistry

Human subcutaneous fibroblasts were seeded in chamber slides at a density of $2.5x10^4$ cells/mL and allowed to grow for 5-15 days. Cultured cells were fixed in 4% paraformaldehyde (PFA) in 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% NaN_3) for 1 h. Primary antibodies, diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton-X, 0.05% NaN_3), were applied [mouse anti-porcine vimentin 1:75 (DAKO); rabbit anti-human collagen I 1:50 (AbDSerotec); mouse anti-human α-smooth muscle actin-FITC 1:250 (Sigma-Aldrich); rabbit anti-human $P2Y_{12}$ 1:100 (Alomone); rabbit anti-human Cx43 1:600 and rabbit anti-human Panx1 1:1000 (Abcam)] and the slides incubated overnight at 4ºC. After incubation, cells were washed 3 times in PBS 1X (10 minutes each). The Alexa Fluor 488 1:1500 (anti-rabbit) and Alexa Fluor 568 1:1500 (anti-mouse) secondary antibodies (Molecular Probes, Invitrogen) were diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton-X) and applied for 1 h protected from light. A last wash was performed with PBS 1X and glass slides were mounted with VectaShield medium and stored at 4ºC. For negative controls, the secondary antibodies were applied without pre-incubation with primary antibodies. A positive control for α-smooth muscle actin (SMA) was performed with cardiac myofibroblasts isolated from Wistar rats (Charles River, Barcelona) using a similar procedure as previously described for human subcutaneous

fibroblasts. Observations were performed and analyzed with a laser-scanning confocal microscope (49).

SDS-PAGE and Western blotting

Fibroblasts were homogenized in a lysis buffer with the following composition: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS and a protease inhibitor cocktail. Protein content of the samples was evaluated using the BCA protein assay kit according to the manufacturer's instructions (Pierce). Samples were solubilized in SDS reducing buffer (0.125 mM Tris-HCl, 4% SDS, 0.004% bromphenol blue, 20% glycerol, and 10% 2-mercaptoethanol, pH 6.8 at 70ºC for 10 minutes), subjected to electrophoresis in 10% SDS-polyacrylamide gels and electrotransferred onto PVDF membranes (MilliPore). Protein loads were 25 μg for Panx1 and 15 μg for Cx43. The membranes were blocked for 1 h in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 + 5% BSA. Membranes were subsequently incubated with rabbit anti-human Panx1 1:250 (Novex, Life Technologies) and rabbit anti-human Cx43 1:6000 (Abcam) in the above blocking buffer overnight at 4ºC. Membranes were washed three times for 10 minutes in 0.1% Tween 20 in TBS and then incubated with donkey anti-rabbit IgG (HRP) 1:30000 (Abcam) secondary antibody, for 60 minutes at room temperature. For comparison purpose, the membranes were also incubated with rabbit anti-human β-tubulin 1:2500 (Abcam) antibody following the procedures described above. Membranes were washed three times for 10 minutes and antigen-antibody complexes were visualized by chemiluminescence with an ECL reagent using the ChemiDoc MP imaging system (Bio-Rad Laboratories).

Reagents and materials

2-Mercaptoethanol, 2-octanol, 8-phenyltheophylline (8-PT), adenosine 5′-diphosphate sodium salt (ADP), adenosine 5′-monophosphate sodium salt (AMP), adenosine 5′-triphosphate sodium salt (ATP), amphotericin B, apyrase, bovine serum albumin (BSA), bradykinin (BK), brefeldin A (BFA), bromphenol blue, carbenoxolone (CBX), Dulbecco's Modified Eagle's Medium (DMEM), ethylene diaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), fetal bovine serum (FBS), glycerol, mefloquine (MFQ), 5′-(N-ethylcarboxamide)adenosine (NECA), penicillin/streptomycin, phosphate

buffered saline system (PBS), protease inhibitor cocktail, quinacrine dihydrochloride, sodium deoxycholate, sodium dodecyl sulfate (SDS), trypsin, Tween 20, and type I collagenase were purchased from Sigma-Aldrich. ^{10}Panx, 2-(propylthio)adenosine-5'-O-(β,γ-difluoro methylene) triphosphate tetrasodium salt (AR-C 66096), 2-[(2-chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridine carboxaldehyde disodium salt (MRS 2211), 2′-deoxy-N6-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS 2179), icatibant (HOE-140), R715, sodium metatungstate (POM-1) and thapsigargin were obtained from Tocris Cookson Inc.. Bafilomycin A1 was from WAKO Chemicals and Triton™ X-100 was obtained from Merck. BFA, MFQ, NECA and thapsigargin were prepared in dimethyl sulfoxide (DMSO). All other drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of DMSO at the maximal concentration used (0.05% v/v), were observed. The pH of the solutions did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

Presentation of data and statistical analysis

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

RESULTS

Characterization of human fibroblast cells in culture

Cultured cells obtained from human subcutaneous connective tissue through the explant technique are elongated and exhibited a spindle-shape morphology, which is characteristic of fibroblasts (94). At the time that functional experiments were conducted, all cells exhibited positive immunoreactivity against fibroblast-cell markers, vimentin (Figure 2.2.1Ai, red) and type I collagen (Figure 2.2.1Ai, green) (96), and no specific staining was developed against stress fibers

containing α-smooth muscle actin (SMA-FITC, Figure 2.2.1Aii). Negative controls, in which cells were incubated only with the secondary antibodies Alexa Fluor 488 (green) and Alexa Fluor 568 (red), are shown in Figure 2.2.1Aiii. For comparison purposes, Figure 2.2.1Aiv illustrates a positive control of SMA-FITC obtained in rat cardiac myofibroblasts where SMA-immunoreactivity exhibits a clear filamentary pattern (Figure 2.2.1Aiv), which was not observed in human subcutaneous fibroblasts (Figure 2.2.1Aii).

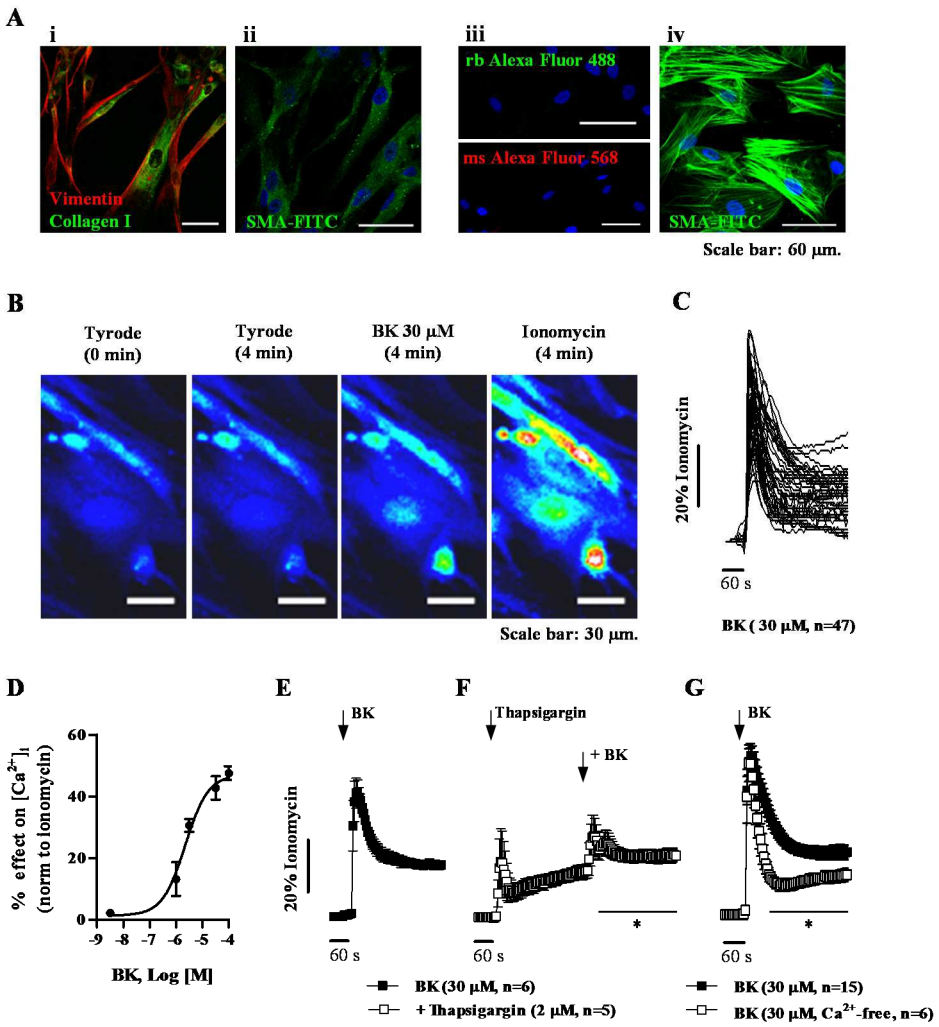

Figure 2.2.1. Bradykinin stimulates the release of intracellular Ca^{2+} stores and Ca^{2+} influx from the extracellular space. Panel **A** shows immunoreactivity of cells cultured from explants of human subcutaneous tissue against fibroblast-cell markers, vimentin (red, **Ai**) and type I collagen (green, **Ai**), and α-smooth muscle actin (SMA-FITC, green, **Aii**). Negative controls, in which cells were incubated only with secondary antibodies, Alexa Fluor 488 (green) and Alexa Fluor 568 (red), are shown for comparison purposes (**Aiii**); a positive control of SMA-FITC immunoreactivity in rat cardiac myofibroblasts is also shown (green, **Aiv**). Cell nuclei are stained with DAPI (blue); scale bar 60 μm. Panel **B** illustrates intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations in cultured human subcutaneous fibroblasts loaded with the fluorescent calcium indicator, Fluo-4 NW (2.5 μM, see Experimental procedures) obtained in the absence and in the presence of bradykinin (BK, 30 μM). Changes in fluorescence were detected in the time-lapse mode with a confocal microscope. Calibration to the maximal calcium load produced by ionomycin (5 μM, 100% response) is also shown for comparison. Image scale bars: 30 μm. Panel **C** shows that the kinetics of BK-induced $[Ca^{2+}]_i$ signals differed slightly between cells of a given population. Panel **D** depicts the concentration-response curve of $[Ca^{2+}]_i$ oscillations produced by BK (0.003-100 μM). Panels **E**, **F** and **G**, represent $[Ca^{2+}]_i$ oscillations produced by BK (30 μM) applied in the absence (**E**) and in the presence of the selective endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (2 μM, **F**), and after removal of extracellular Ca^{2+} (Ca^{2+}-free medium plus EGTA, 100 μM, **G**). Black arrows indicate the time of drugs application. Each point represents pooled data from an *n* number of experiments. The vertical bars represent S.E.M.. *p < 0.05 represent significant differences from BK (30 μM) alone.

Bradykinin, via B_2 receptors, stimulates the release of intracellular Ca^{2+} stores and Ca^{2+} influx from the extracellular space

Bradykinin (0.001-100 μM) caused prominent intracellular Ca^{2+} ($[Ca^{2+}]_i$) rises in human subcutaneous fibroblasts (Figure 2.2.1). Global changes in $[Ca^{2+}]_i$ were monitored with a multidetection microplate reader after pre-incubation of the cells with the calcium sensitive dye, Fluo-4 NW; in some instances, single-cell $[Ca^{2+}]_i$ imaging was also performed using a laser scanning confocal microscope in the time-lapse mode (Figure 2.2.1B and 2.2.1C). The effect of bradykinin (0.001-100 μM) was dependent on the concentration (Figure 2.2.1D); significant ($p < 0.05$) $[Ca^{2+}]_i$ rises were observed at concentrations higher than 1 μM. Bradykinin typically produced a biphasic response (Figure 2.2.1C); at 30 μM concentration, $[Ca^{2+}]_i$ raised abruptly to a peak that attained 44 ± 2% of the maximal calcium load produced by ionomycin (5 μM, 100% response), then declined to a plateau of elevated $[Ca^{2+}]_i$ which remained fairly constant until drug washout (Figure 2.2.1E). Bradykinin (30 μM) produced negligible changes in $[Ca^{2+}]_i$ in the presence of the selective inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (2 μM, $n = 5$), which is known to deplete intracellular Ca^{2+} stores following a transient (< 2 min) rise of $[Ca^{2+}]_i$ levels (97) (Figure 2.2.1F). Removal of external Ca^{2+} (plus EGTA, 100 μM, $n = 6$) significantly ($p < 0.05$) attenuated the plateau phase, but the magnitude of the peak was kept almost unchanged (Figure 2.2.1G). It, thus, appears that in human subcutaneous fibroblasts the initial transient component must be caused by intracellular Ca^{2+} release from internal stores. The sustained plateau of elevated $[Ca^{2+}]_i$ results from Ca^{2+} entry through the plasma membrane in response to depletion of Ca^{2+} stores and/or through the concurrent activation of other membrane-bound receptors, namely P2 purinoceptors. This pattern is consistent with previous findings in the literature regarding bradykinin-induced $[Ca^{2+}]_i$ responses in several cell types (138,139), including human fibroblasts of the foreskin (140).

Bradykinin-induced $[Ca^{2+}]_i$ rise in human subcutaneous fibroblasts was concentration-dependently attenuated by the selective B_2 receptor antagonist, HOE-140 (1 and 10 μM, $n = 7$) (Figure 2.2.2B and 2.2.2C), whereas selective blockade of the B_1 receptor with R715 (1 μM, $n = 4$) was without effect (Figure 2.2.2A). At the highest concentration (10 μM), HOE-140 significantly ($p < 0.05$) decreased, but did not completely block, the late phase of bradykinin-induced $[Ca^{2+}]_i$ response given that the peptide was used in a concentration (30 μM) near

that necessary for saturation of B_2 receptors in these cells (see Figure 2.2.1D). On their own, the two antagonists were devoid of any significant effect.

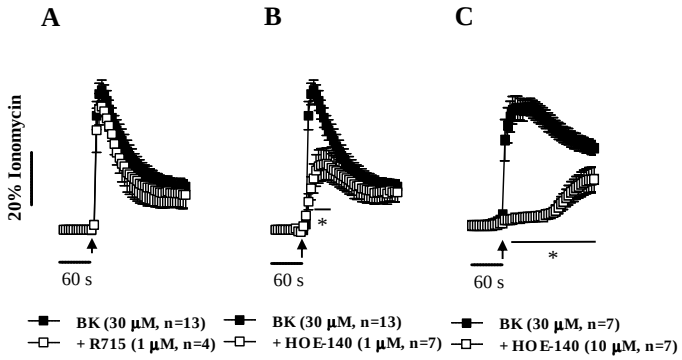

Figure 2.2.2. Bradykinin-induced $[Ca^{2+}]_i$ rise in human subcutaneous fibroblasts depend on B_2 receptor activation. Calcium oscillations in human fibroblasts of the subcutaneous tissue stimulated with bradykinin (BK, 30 μM) were tested after pretreatment of the cells with selective B_1 and B_2 receptor antagonists, respectively R715 (1 μM, **A**) and HOE-140 (1 μM, **B**; 10 μM, **C**). Cells were pre-incubated with the cell-permeant fluorescent calcium indicator, Fluo-4 NW (2.5 μM, see Experimental procedures). Changes in fluorescence were detected using a microplate reader. Intracellular Ca^{2+} transients were calibrated to the maximal calcium load produced by ionomycin (5 μM, 100% response). Black arrows indicate the time of drugs application. No changes in baseline fluorescence were observed after application of the antagonists. Each point represents pooled data from an *n* number of experiments. The vertical bars represent S.E.M.. *$p < 0.05$ represent significant differences from BK (30 μM) alone.

Bradykinin induces ATP release from human subcutaneous fibroblasts: involvement of intracellular Ca^{2+} stores

The release of ATP from human subcutaneous fibroblasts in culture was inferred from destaining of cells loaded with quinacrine, an ATP-binding intracellular fluorescent dye, by confocal microscopy in the time-lapse mode. Bradykinin (30 μM, $n = 35$) increased ($p < 0.05$) fluorescence intensity decay of cells loaded with quinacrine as compared to the control situation in which the cells were challenged with the Tyrode's solution (Figure 2.2.3A and 2.2.3B). Quinacrine destaining of cells exposed to bradykinin was more evident at the periphery than near the nucleus (Figure 2.2.3A). Confirmation that human subcutaneous fibroblasts release ATP in response to bradykinin (30 μM, $n = 4$) was obtained by measuring the luminescence of the medium before and after bradykinin application to cells incubated with luciferin-luciferase (Figure 2.2.3C). Results demonstrate that ATP release peaked at 30 s after bradykinin (30 μM) application and was kept fairly constant during the 4-min drug application.

Pretreatment with thapsigargin (2 μM, $n = 6$) significantly ($p < 0.05$) attenuated bradykinin-induced quinacrine destaining (Figure 2.2.3Di). In contrast,

removal of extracellular Ca^{2+} (plus EGTA, 100 μM, n = 8) from the incubation medium did not affect significantly ($p > 0.05$) the fluorescence intensity decay of quinacrine stained ATP granules (Figure 2.2.3Dii). These observations indicate that Ca^{2+} recruitment from thapsigargin-sensitive internal stores is required for the release of ATP induced by bradykinin.

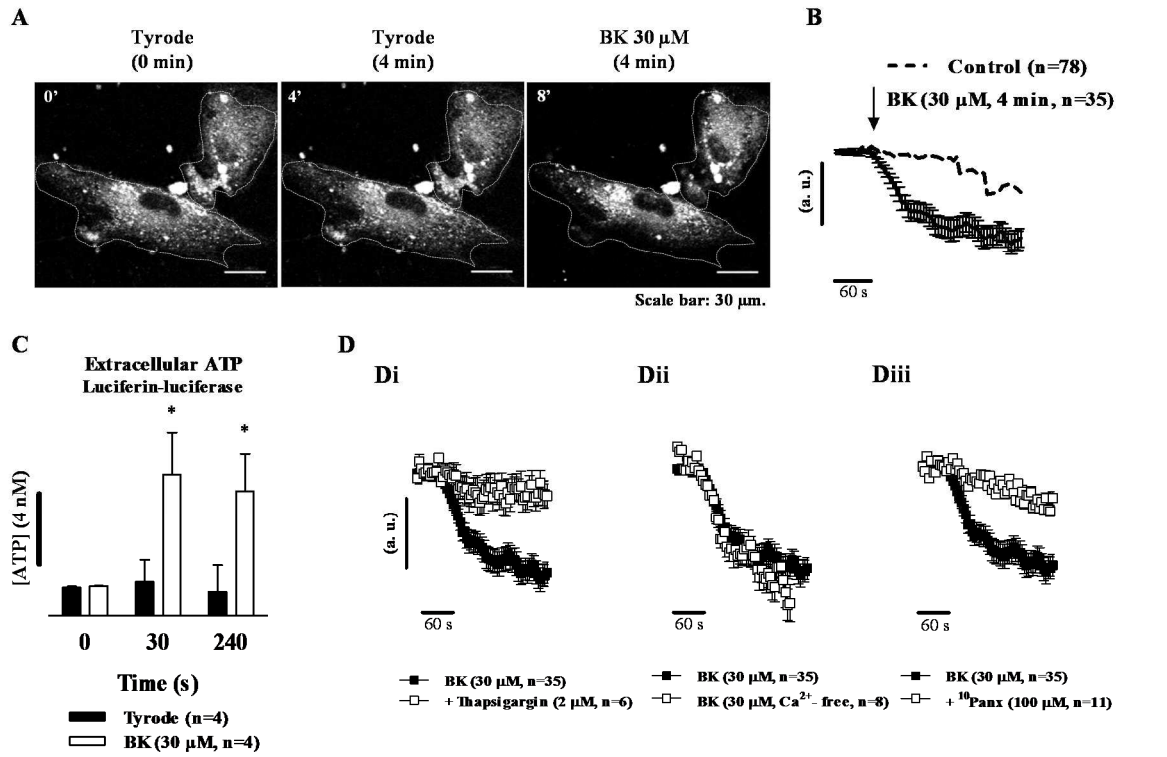

Figure 2.2.3. Bradykinin elicits ATP release from human fibroblasts by a mechanism depending on intracellular Ca^{2+} mobilization. Panels **A**, **B** and **D** represent cells loaded with quinacrine (30 μM, a fluorescent dye that specifically binds ATP), for 60 min at 37ºC. ATP release was detected by single-cell confocal microscopy in the time-lapse mode measuring the fluorescence intensity decay 4 min after bradykinin (BK, 30 μM, **A** and **B**) application as compared to the control situation, in which only Tyrode's solution was applied (**A**). Panel **D**, shows the effect of BK (30 μM) after pretreatment of the cells with the selective endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (2 μM, **Di**), and after removal of extracellular Ca^{2+} (Ca^{2+}-free medium plus EGTA, 100 μM, **Dii**); the effect of BK (30 μM) in the presence of the selective Panx1 inhibitor, ^{10}Panx (100 μM, **Diii**), is also shown. Image scale bars: 30 μm. Graphs show quinacrine fluorescence decay (arbitrary units, a.u.) plotted versus time in the presence of Tyrode's solution (**B**), thapsigargin (2 μM, **Di**), Ca^{2+}-free medium (**Dii**) and ^{10}Panx (100 μM, **Diii**). Black arrows indicate the time of drugs application. Each point represents pooled data from an n number of cells. The vertical bars represent S.E.M.. Panel **C**, shows the ATP content in human subcutaneous fibroblast cultures at given time intervals (0-240 seconds) in the presence of BK (30 μM, open bars) as compared to the control condition where only Tyrode's solution was applied (closed bars). Relative luminescence units (RLUs) were calibrated using a 4 nM ATP standard (left hand-side black vertical bar). Each bar represents pooled data from an n number of experiments. The vertical bars represent S.E.M.. *$p < 0.05$ represent significant differences from BK (30 μM) alone.

ATP release via connexin and pannexin-1 hemichannels contributes to bradykinin-induced $[Ca^{2+}]_i$ mobilization

Among other mechanisms, hemichannels containing connexins (Cx) and pannexin-1 (Panx1) are now widely accepted as putative mediators of ATP translocation to the extracellular milieu in non-excitable cells. The expression of Cx43 is characteristic of fibroblasts from multiple tissue origins (78,98). Using immunofluorescence confocal microscopy (Figure 2.2.4Ai) and Western blot analysis (Figure 2.2.4Aii), we demonstrated that fibroblasts of the human subcutaneous tissue in culture express anti-Panx1 (~50 kDa) immunoreactivity in addition to the expected Cx43 (43 kDa). Panx1 and Cx43 are both expressed in relatively high density as compared to β-tubulin standard protein levels (Figure 2.2.4Aiii).

These findings prompted us to test whether bradykinin-induced $[Ca^{2+}]_i$ oscillations in human subcutaneous fibroblasts depend on the release of ATP via hemichannels using subtype selective connexin and pannexin-1 inhibitors. Inhibition of Cx36- and Cx50-containing hemichannels with mefloquine (99,100) (MFQ, 3 μM, n = 8) was devoid of effect on bradykinin-induced $[Ca^{2+}]_i$ rise (Figure 2.2.4Bi). On the other hand, 2-octanol (1 mM, n = 6), which blocks Cx43, Cx46 and Cx50 hemichannels (100,101) (Figure 2.2.4Bii), and carbenoxolone (CBX, 300 μM, n = 5), a non-selective inhibitor of connexins Cx26, Cx30, Cx32, Cx43 and Cx46, which also blocks Panx1-containing hemichannels (101) (Figure 2.2.4Biii), significantly ($p < 0.05$) attenuated the $[Ca^{2+}]_i$ response induced by bradykinin (30 μM). Interestingly, the selective Panx1 mimetic inhibitory peptide, ^{10}Panx (102) (100 μM, n = 6), also decreased the bradykinin-induced $[Ca^{2+}]_i$ response (Figure 2.2.4Biv). CBX (300 μM) was the most effective of the three inhibitors, probably because it has a broad inhibitory spectrum blocking equally well connexin and Panx1 containing hemichannels. Coincidently or not, 2-octanol (1 mM), CBX (300 μM) and ^{10}Panx (100 μM), were more effective in depressing the plateau phase of bradykinin (30 μM) response (Figures 2.2.4Bii-4Biv), although 2-octanol (1 mM) and CBX (300 μM) also inhibited ($p < 0.05$) the fast $[Ca^{2+}]_i$ rise induced by the peptide. Confocal microscopy studies demonstrated that ^{10}Panx (100 μM) also attenuated ($p < 0.05$) bradykinin (30 μM)-induced ATP release from human fibroblasts loaded with quinacrine (see Figure 2.2.3Diii).

In order to test if bradykinin-induced $[Ca^{2+}]_i$ oscillations involved nucleotides-release by exocytosis we used the vesicular transport inhibitor, brefeldin A (BFA,

Figure 2.2.4Bv), and the specific inhibitor of H^+-ATPases of the vacuolar type, bafilomycin A1 (Baf A1, Figure 2.2.4Bvi). No statistical significant ($p > 0.05$) differences were found in $[Ca^{2+}]_i$ oscillations produced by bradykinin (30 μM) in the absence and in the presence of BFA (20 μM, $n = 4$) and Baf A1 (3 μM, $n = 7$). Confocal microscopy experiments with primary cultures of rat subcutaneous fibroblasts loaded with the calcium sensitive dye, Fluo-4 NW, and then incubated with FM4-64, a membrane-selective fluorescent dye, were used to evaluate vesicle endocytosis and exocytosis in the time lapse mode (136,137). Results depicted in Figure 2.2.4Ci, show that bradykinin (30 μM)-induced $[Ca^{2+}]_i$ oscillations were not accompanied by measurable changes in FM4-64 fluorescence signals in the same cells (Figure 2.2.4Cii). Ionomycin (5 μM) increased moderately FM4-64 fluorescence labeling because plasma membrane modifications (e.g. membrane protrusions, microvesiculation, blebbing) are likely to occur in the presence of the Ca^{2+} ionophore. Overall, our findings suggest that ATP release via connexins (most probable Cx43) and Panx1 hemichannels, rather than by vesicle exocytosis, may contribute importantly to the plateau phase of bradykinin-induced $[Ca^{2+}]_i$ response in fibroblasts cultured from the human subcutaneous connective tissue.

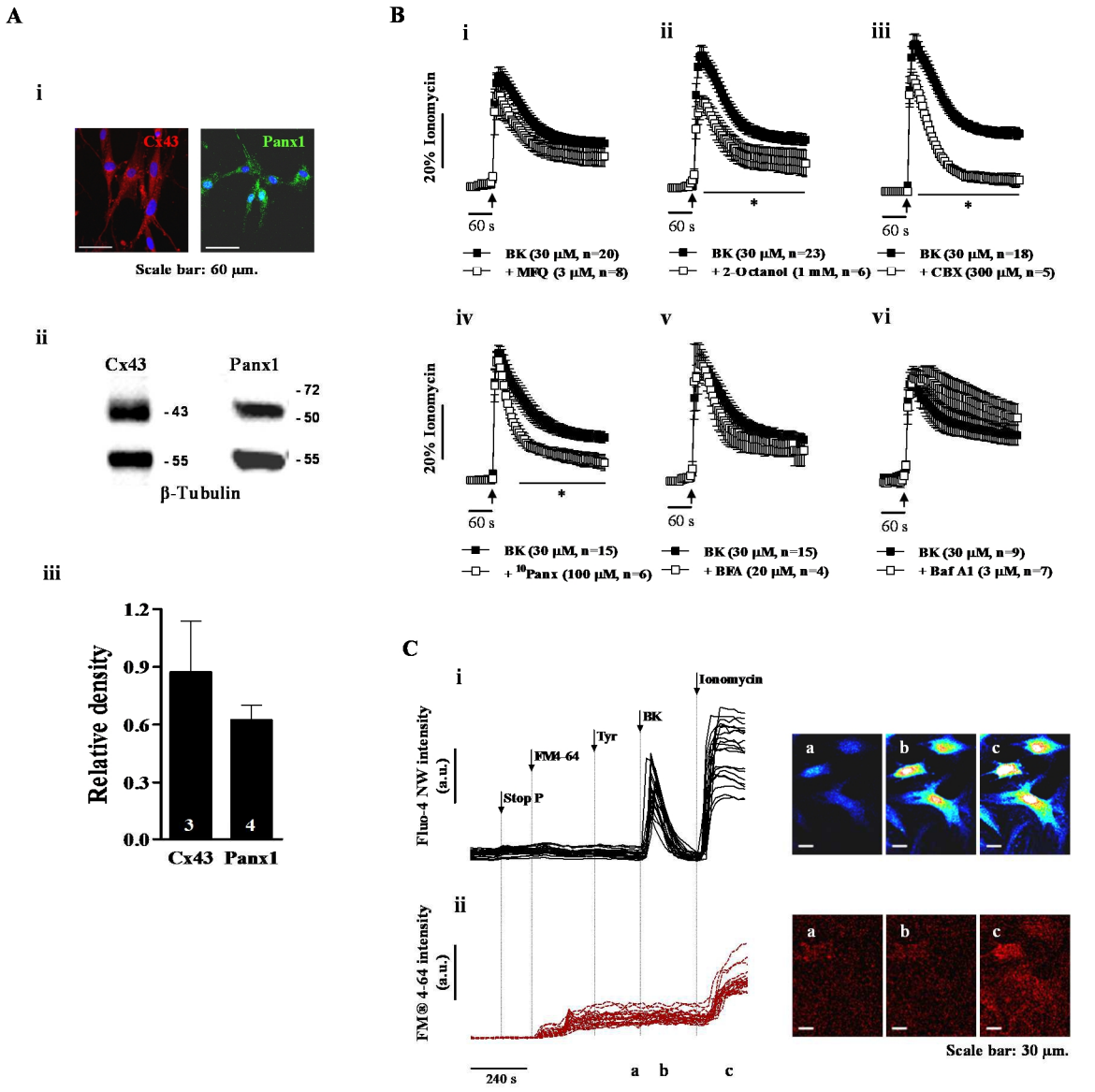

Figure 2.2.4. Bradykinin-induced $[Ca^{2+}]_i$ signals in human subcutaneous fibroblasts involve connexin and pannexin-1 containing hemichannels opening. Panel **A** shows representative confocal micrographs (**Ai**) and blots (**Aii**) of Cx43 and Panx1 hemichannels immunoreactivity in cultured human subcutaneous fibroblasts from 3-4 individuals. Image scale bars: 60 µm. β-tubulin was used as a reference protein to determine the relative density of Cx43 and Panx1 immunoblots (**Aiii**). Panel **B** shows the effect of bradykinin (BK, 30 µM) tested in the presence of inhibitors of connexin and/or Panx1 hemichannels, namely mefloquine (MFQ, 3 µM, **Bi**), 2-octanol (1 mM, **Bii**), carbenoxolone (CBX, 300 µM, **Biii**) and ^{10}Panx (100 µM, **Biv**). The effects of the vesicular transport inhibitor, brefeldin A (BFA, 20 µM, **Bv**), and of the specific inhibitor of vacuolar H^+-ATPases, bafilomycin A1 (Baf A1, 3 µM, **Bvi**), are also shown for comparison. Cells were pre-incubated with the cell-permeant fluorescent calcium indicator, Fluo-4 NW (2.5 µM, see Experimental procedures). Changes in fluorescence were detected using a microplate reader. $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Black arrows indicate the time of drugs application. None of the inhibitors significantly change baseline fluorescence when applied alone. Each point represents pooled data from an *n* number of experiments. The vertical bars represent S.E.M.. *p < 0.05 represent significant differences from BK (30 µM) alone. Panel **C** shows representative traces of single-cell $[Ca^{2+}]_i$ oscillations in rat subcutaneous fibroblasts loaded with Fluo-4 NW (**Ci**) obtained in parallel with the incorporation of the membrane-selective fluorescent dye, FM4-64 (**Cii**), used to evaluate vesicle endocytosis and exocytosis in cells. Changes in fluorescence were detected in the time-lapse mode using a laser-scanning confocal microscope. Vertical lines indicate the time of drugs application. Fluorescence confocal micrographs were obtained at the indicated time points (a, b and c). Image scale bars: 30 µm.

The plateau phase of bradykinin $[Ca^{2+}]_i$ recruitment is partially dependent on the activation of ADP-sensitive $P2Y_{12}$ purinoceptors

Extracellular inactivation of ATP directly into AMP with apyrase (NTPDase1 or CD39, 2 U/mL, n = 8) reduced significantly ($p < 0.05$) the plateau phase of bradykinin (30 μM, n = 17) response, while keeping fairly conserved the magnitude of the initial $[Ca^{2+}]_i$ rise (Figure 2.2.5Ai). Surprisingly, inhibition of membrane-bound NTPDases with POM-1 (141) (20 μM, n = 11) also decreased the plateau phase of bradykinin-induced $[Ca^{2+}]_i$ response in human subcutaneous fibroblasts (Figure 2.2.5Aii). The result obtained with POM-1 (20 μM) was confirmed when we tested the effect of bradykinin on $[Ca^{2+}]_i$ oscillations in the absence of Mg^{2+}, an ion that must be present in millimolar concentration in the extracellular fluid for maximum activity of ectonucleotidases (36) (Figure 2.2.5Aiii). These findings provide the first evidence that the plateau phase of bradykinin-induced $[Ca^{2+}]_i$ accumulation by human subcutaneous fibroblasts requires the release of ATP and its subsequent conversion into other biologically active metabolites, most probably ADP, by ectonucleotidases.

The kinetics of the extracellular catabolism of adenine nucleotides (ATP, ADP and AMP) and formation of metabolites in fibroblasts cultured from the human subcutaneous tissue is shown in Figure 2.2.5B. Average half-life times of ATP, ADP and AMP were respectively 12.6 ± 4.2 min, 27.0 ± 1.6 min and 1.5 ± 0.2 min (n = 4 observations from two individuals), when the substrates were used in a 3 μM concentration. ATP (3 μM) was sequentially metabolized into ADP, adenosine (ADO), inosine (INO) and hypoxanthine (HX) (Figure 2.2.5B). AMP (3 μM) was rapidly and sequentially converted into ADO and INO respectively by ecto-5'-nucleotidase (CD73) and adenosine deaminase (ADA), which might explain why AMP accumulation was almost negligible when ATP (3 μM) and ADP (3 μM) were used as substrates.

The analysis of the corresponding half-life time values clearly indicates that the extracellular catabolism of ADP into AMP is the rate-limiting step to generate ADO from exogenously added adenine nucleotides in cultured human subcutaneous fibroblasts. Therefore, transient accumulation of ADP in the cultures is in favor of a preferential activation of ADP-sensitive P2Y purinoceptors. To investigate the contribution of ADP-sensitive P2Y purinoceptors activation to bradykinin (30 μM)-induced $[Ca^{2+}]_i$ response in human subcutaneous fibroblasts, we tested its effect in the presence of selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors

antagonists (Figure 2.2.5C). Selective blockade of the $P2Y_{12}$ receptor with AR-C 66096 (0.1 μM, n = 6) significantly ($p < 0.05$) attenuated the plateau phase of $[Ca^{2+}]_i$ rise caused by bradykinin (30 μM, n = 20) without much affecting the magnitude of the initial $[Ca^{2+}]_i$ rise (Figure 2.2.5Cii). No significant differences ($p > 0.05$) were observed in the presence of MRS 2179 (0.3 μM, n = 10) and MRS 2211 (10 μM, n = 10) which selectively antagonize $P2Y_1$ and $P2Y_{13}$ receptors, respectively (Figure 2.2.5Ci and 2.2.5Ciii). None of these antagonists modified *per se* $[Ca^{2+}]_i$ in human subcutaneous fibroblasts. The expression of the $P2Y_{12}$ receptor in cultured human subcutaneous fibroblasts was confirmed by immunocytochemistry (Figure 2.2.5D).

Adenine nucleotides, but not adenosine, increase the influx of Ca^{2+} in human subcutaneous fibroblasts

Changes in $[Ca^{2+}]_i$ in human subcutaneous fibroblasts treated with adenine nucleotides (1 mM) in the presence and in the absence of extracellular Ca^{2+} are shown in Figure 2.2.6A. Exogenous application of ATP and ADP (1 mM) transiently increased intracellular $[Ca^{2+}]_i$ reaching, respectively, 43 ± 4% (n = 8) and 25 ± 8% (n = 3) of the maximal calcium load caused by ionomycin (5 μM, 100% response) within 20 s from drug application. In Ca^{2+}-free medium (plus EGTA, 100 μM), ATP- and ADP-induced $[Ca^{2+}]_i$ transients were partially but significantly ($p < 0.05$) attenuated (Figure 2.2.6A).

Adenosine (ADO) is a recognized anti-nociceptive agent (see *e.g.* (142)) and plays prominent roles in fibroblasts proliferation and tissue remodeling in the skin, liver and lung (66), yet contradictory effects of the nucleoside have been described on cardiac fibroblasts (63,64). Given the high amounts of ADO accumulating in cultured fibroblasts of the human subcutaneous tissue as a result of the extracellular catabolism of released adenine nucleotides (see Figure 2.2.5), we sought to investigate the contribution of the nucleoside to bradykinin-induced Ca^{2+} signals in these cells. To this end, we used the enzymatically stable adenosine analogue, 5′-(N-ethylcarboxamide) adenosine (NECA), which non-selectively activates all four adenosine receptor subtypes in the submicromolar range. NECA (10 μM, n = 6) had a negligible effect on $[Ca^{2+}]_i$ in human subcutaneous fibroblasts when it was applied either alone (Figure 2.2.6Bii) or in the presence of bradykinin (30 μM, n = 5) (Figure 2.2.6Biii). No statistical significant ($p > 0.05$) difference was found in $[Ca^{2+}]_i$ oscillations caused by

bradykinin (30 μM) in the presence of NECA (10 μM, *n* = 6) (Figure 2.2.6Bii) as compared to the control situation (Figure 6Bi). Moreover, pretreatment of the cells with the non-selective adenosine receptor antagonist, 8-phenyltheophylline (8-PT, 2.5 μM, *n* = 5), was devoid of effect on bradykinin-induced $[Ca^{2+}]_i$ rise (Figure 2.2.6C), suggesting that adenosine has a minor effect on Ca^{2+} signaling operated by bradykinin in human subcutaneous fibroblasts.

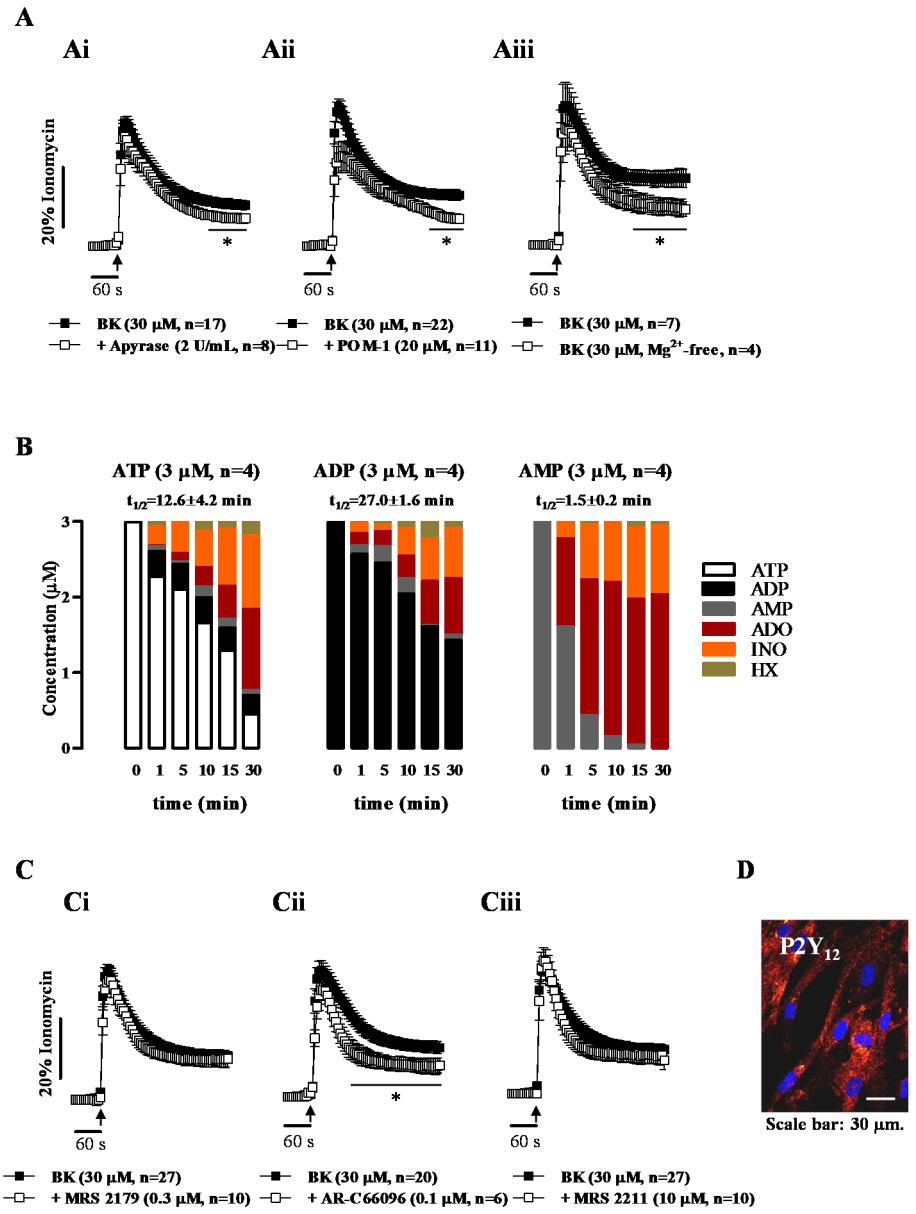

Figure 2.2.5. Bradykinin-induced $[Ca^{2+}]_i$ mobilization is partially dependent on the activation of $P2Y_{12}$ purinoceptor. Panel **A** shows the effect of bradykinin (BK, 30 µM) after pretreating human subcutaneous fibroblasts with apyrase (2 U/mL, **Ai**), which catabolizes ATP/ADP into AMP, and after inhibition of ectonucleotidases, with POM-1 (20 µM, **Aii**) or after removing Mg^{2+} from the incubation fluid (**Aiii**). Panel **C** shows the effects of BK (30 µM) in the absence/presence of selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor antagonists, respectively MRS 2179 (0.3 µM, **Ci**), AR-C 66096 (0.1 µM, **Cii**) and MRS 2211 (10 µM, **Ciii**). Cells were pre-incubated with the cell-permeant fluorescent calcium indicator, Fluo-4 NW (2.5 µM, see Experimental procedures). $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Black arrows indicate the time of drugs application. No changes in baseline fluorescence were observed after application of the modulators. Each point represents pooled data from an *n* number of experiments. The vertical bars represent S.E.M.. $*p < 0.05$ represent significant differences from BK (30 µM) alone. Panel **B** illustrates the time course of the extracellular catabolism of adenine nucleotides in human subcutaneous fibroblasts grown in culture for 11 days. ATP, ADP or AMP (3 µM) were added to the culture medium at time zero; samples (75 µL) were collected at indicated times. Each sample was analyzed by HPLC to separate and quantify ATP (white), ADP (black), AMP (grey), adenosine (ADO, red), inosine (INO, orange) and hypoxanthine (HX, green). Each point represents pooled data from two individuals; 2 replicas were performed for each individual. The calculated half-life time ($t_{½}$) for each initial substrate is shown for comparison. Panel **D** shows immunoreactivity of human subcutaneous fibroblasts against the $P2Y_{12}$ receptor; shown image is representative of three independent experiments. Image scale bar is 30 µm.

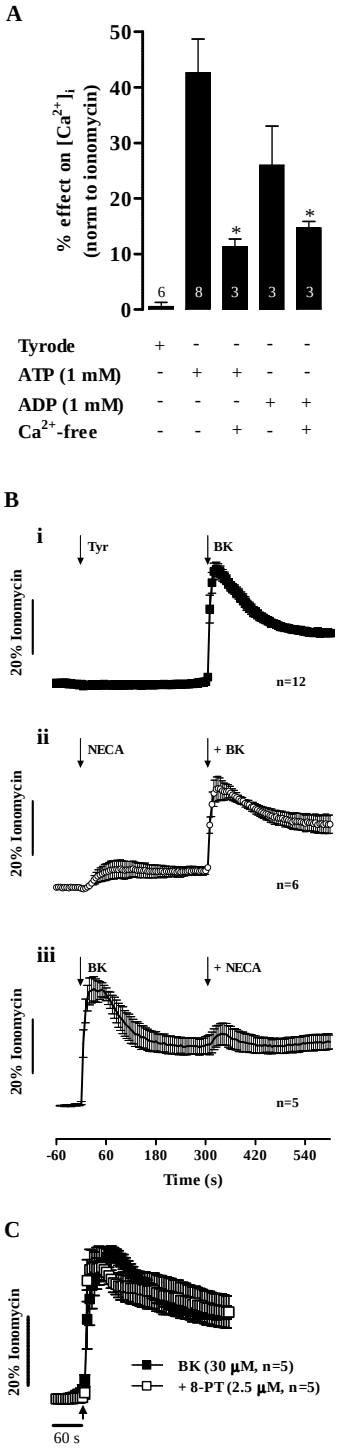

Figure 2.2.6. Adenine nucleotides, but not adenosine, increase Ca^{2+} influx from the extracellular space in human subcutaneous fibroblasts. In panel **A**, human subcutaneous fibroblasts were incubated with 1 mM ATP or ADP in control conditions and in Ca^{2+}-free medium (plus EGTA, 100 µM). Each bar represents pooled from an *n* number of experiments. The vertical bars represent S.E.M.. *$p < 0.05$ represent significant differences compared with the effect of ATP or ADP alone. Panel **B** illustrates $[Ca^{2+}]_i$ oscillations caused by NECA (10 µM), an enzymatically stable non-selective adenosine receptor agonist, compared with the effect of bradykinin (BK, 30 µM) in fibroblasts of the human subcutaneous tissue. NECA (10 µM) and BK (30 µM) were applied cumulatively in sequence (**Bii**) and in the reverse mode (**Biii**). Panel **C** shows BK (30 µM)-induced $[Ca^{2+}]_i$ oscillations in the absence and in the presence of a non-selective adenosine receptor antagonist, 8-PT (2.5 µM). Cells were pre-incubated with the cell-permeant fluorescent calcium indicator, Fluo-4 NW (2.5 µM, see Experimental procedures). Changes in fluorescence were detected using a microplate reader. $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM, 100% response). Black arrows indicate the time of drugs application. No changes in baseline fluorescence was observed after application of 8-PT (2.5 µM). Each point represents pooled data from an *n* number of experiments. The vertical bars represent S.E.M..

DISCUSSION

Bradykinin is produced within the interstitium of most tissues and plays an important role in normal and pathological conditions, being considered an important inflammatory pain mediator (143) that is associated with chronic musculoskeletal pain syndromes (144,145). Recent findings have shown that in the central nervous system bradykinin triggers astrocyte-neuron signaling via glutamate release followed by NMDA receptors activation (146). In peripheral tissues, bradykinin receptors have already been described in sensory neurons of dorsal root ganglia (DRG), where it exerts direct and indirect effects via neuronal excitation and threshold modulation by the release of excitatory signaling molecules, respectively (22). To the best of our knowledge, this is the first report demonstrating that fibroblasts isolated from the human subcutaneous connective tissue respond to bradykinin by releasing ATP into the extracellular medium through the activation of B_2 receptors, which are constitutively expressed in most of the tissues exhibiting bradykinin sensitivity. Although our experiments were conducted in non-stressful conditions, the involvement of inducible B_1 receptors mediating bradykinin effects in human subcutaneous fibroblasts cannot be ruled out (147). Bradykinin-induced ATP release from these cells was demonstrated by two distinct experimental approaches: the luciferin-luciferase bioluminescence assay and ATP-binding quinacrine dye destaining by time-lapse confocal microscopy. Our data also suggest that bradykinin-induced ATP release from human subcutaneous fibroblasts requires Ca^{2+} recruitment from thapsigargin-sensitive internal stores and occurs independently of extracellular Ca^{2+} by electrodiffusional translocation of the nucleotide via hemichannels containing connexins (most probably Cx43) and Panx1.

In several cell types (138,139), including human fibroblasts of the foreskin (140), bradykinin elicits an abrupt rise in $[Ca^{2+}]_i$ to a peak that depends on Ca^{2+} recruitment from internal stores, which then declines to a plateau that is sustained by Ca^{2+} entry from the extracellular compartment. The long lasting elevation of $[Ca^{2+}]_i$ seen after the initial $[Ca^{2+}]_i$ peak probably results from a receptor- or second messenger-operated Ca^{2+} current activated by bradykinin, rather than via a capacitative pathway (140). Here, we demonstrate for the first time that ADP-sensitive $P2Y_{12}$ purinoceptors may be, at least in part, responsible for the Ca^{2+} influx that is characteristic of the plateau phase of elevated $[Ca^{2+}]_i$ caused by bradykinin in human subcutaneous fibroblasts. This conclusion is supported by results showing that: [1] human subcutaneous fibroblasts exhibit

$P2Y_{12}$ receptor immunoreactivity, [2] bradykinin induces the release of ATP that is subsequently hydrolyzed into ADP by membrane-bound ectonucleotidases, [3] ADP has a tendency to accumulate transiently in the cultures, given that we found that the extracellular catabolism of ADP into AMP is the rate-limiting step of the ectonucleotidase cascade, and, finally, [4] adenine nucleotides-induced $[Ca^{2+}]_i$ rises in human subcutaneous fibroblasts are depend on extracellular Ca^{2+}.

The pathways underlying bradykinin-induced long lasting Ca^{2+} influx resulting from the cooperation with ADP-sensitive $P2Y_{12}$ purinoceptors need to be further investigated. Interestingly, bradykinin causes a rapid translocation of all protein kinase C isoforms from the cytosol to the plasma membrane within the time frame (30 s) of ATP appearance in the cultures (128), which may favor the synergism between B_2 and $P2Y_{12}$ receptors activation. The involvement of ADP-sensitive $P2Y_{12}$ receptors in neuropathic pain, has been already reported; authors suggested that this could be due to an unclear mechanism involving non-neuronal cells, such as spinal microglia (148,149) and trigeminal ganglion satellite cells (150). The close proximity between $P2Y_{12}$ receptor-expressing fibroblasts of the subcutaneous tissue and sensory nerve fibers exhibiting numerous ligand-gated receptors (including ionotropic P2X and metabotropic P2Y purinoceptors) implies that adenine nucleotides released from stimulated fibroblasts may alter acute and chronic pain perception. During acute tissue injury, excessive ATP release from damaged fibroblasts, keratinocytes, blood vessels and inflammatory cells may cause pain by activating excitatory purinoceptors on nociceptive sensory nerve endings (149,151-153). Lower levels of ATP released from intact cells in response to mild mechanical and thermal stimulation may participate in normal tactile sensation and also contribute to the spontaneous pain and tactile hypersensitivity that occurs under chronic painful conditions involving the subcutaneous connective tissue when nerve endings become sensitized (151,154). We, now, postulated a new type of interaction involving an autocrine action of ATP, via $P2Y_{12}$ receptors, triggered by the activation of bradykinin B_2 receptors in human subcutaneous fibroblasts. Moreover, increased sensitivity to extracellular ATP has been described in fibroblasts from patients affected with systemic sclerosis (155).

Little is known about the mechanisms upstream the nucleotide release from human fibroblasts despite the importance of connective tissue ATP signaling in the pathogenesis of acute and chronic inflammatory pain (44,77). Multiple nucleotide-releasing pathways have been identified in intact cells, which represent a critical component for the initiation of the purinergic signaling cascade (77).

Experiments designed to manipulate exocytosis of vesicles/organelles containing compartmentalized ATP suggest that it might not represent an important pathway for releasing ATP from human fibroblasts stimulated with bradykinin. Several non-vesicular ATP release mechanisms have been proposed, yet many remain controversial and are complicated by the non-specificity of available inhibitors. Hemichannels possessing connexin and Panx1 subunits represent an important mechanism for the cellular release of ATP. The opening of hemichannels occurs in response to many physiological and pathological situations, including volume regulation, proliferation, calcium wave propagation by extracellular messengers and cell death during metabolic inhibition (156). Using immunofluorescence confocal microscopy and Western blot analysis, we demonstrated that fibroblasts of the human subcutaneous tissue exhibit strong anti-Panx1 immunoreactivity in addition to Cx43 that is characteristic from fibroblasts of other tissue origins (78,98). Moreover, functional data using non-selective connexin inhibitors targeting Cx43 hemichannels (*e.g.* 2-octanol, CBX) strongly depressed the plateau phase of bradykinin-induced $[Ca^{2+}]_i$ response. Because connexin hemichannels are activated by moderate (< 500 nM) $[Ca^{2+}]_i$ elevations, these channels may open in response to bradykinin during the initial $[Ca^{2+}]_i$ rise (157) and contribute to ATP release and to the subsequent purinoceptor-mediated signaling. Closing of connexin containing hemichannels, which unlike Panx1 hemichannels seal when the spike amplitude rises above 500 nM, contributes to shape bradykinin-induced $[Ca^{2+}]_i$ oscillations as demonstrated by the partial reduction of the initial $[Ca^{2+}]_i$ rise of bradykinin in the presence of 2-octanol and CBX.

Considering the relatively high potency of CBX (300 μM) and the fact that this compound also blocks Panx1 containing hemichannels (101), we tested the effect of the selective Panx1 mimetic inhibitory peptide, ^{10}Panx, which also depressed the plateau phase of the bradykinin-induced $[Ca^{2+}]_i$ response in parallel to the decline of ATP release from fibroblasts loaded with quinacrine. Like the effects obtained with apyrase and with the selective $P2Y_{12}$ antagonist, AR-C 66096, inhibition of hemichannels containing Cx43 and Panx1 were more effective in depressing the plateau phase rather than the peak of the bradykinin response. Taken together, data suggest that Cx43 and Panx1 containing hemichannels have a predominant role on ATP release from human subcutaneous fibroblasts stimulated with bradykinin, thereby instigating the regenerative propagation of intracellular Ca^{2+} signals. Our findings are in agreement with the observation that mechanically stimulated cardiac fibroblasts release ATP in a CBX-

sensitive manner, an effect that the authors attributed to Cx43 hemichannels not excluding a possible involvement of Panx1 containing hemichannels (59).

Regardless of whether channel-mediated efflux or vesicle exocytosis comprises the predominant ATP release mechanism, most studies (but not all, see *e.g.* (158)) have identified elevation of cytosolic Ca^{2+} as an important regulator of nucleotide export in different cell model systems. The molecular mechanism by which bradykinin releases ATP through the opening of Cx43 and Panx1 hemichannels in human subcutaneous fibroblasts may be the generation of inositol trisphosphate (IP_3) by phospholipase C and the downstream $[Ca^{2+}]_i$ mobilization from internal stores (114,115). Our data, showing that intracellular Ca^{2+} depletion with thapsigargin impaired quinacrine dye destaining induced by bradykinin is in favor with the hypothesis that Ca^{2+} mobilization is necessary for ATP release in these cells. Further experiments are required to discard the ability of bradykinin, like certain G_q-coupled receptors, to additionally stimulate Rho-GTPase acting to strongly potentiate a Ca^{2+}-activated ATP release pathway (116,117). Seminario-Vidal and col. (2009) (117), demonstrated that Ca^{2+}- and RhoA/Rho kinase-dependent ATP release from thrombin-stimulated A549 lung epithelial cells occurs via connexin or pannexin hemichannels, a pathway that seems to be not competent for ATP release in human astrocytoma cells (116). Given the actions exerted by Rho/Rho kinase on cytoskeleton components (*e.g.* regulating myosin-light chain phosphorylation and actin polymerization), those authors speculated that Rho-promoted membrane-cytoskeletal rearrangements facilitate the insertion of hemichannel subunits within the plasma membrane.

Bradykinin can increase glutamate release from mouse astrocytes through volume-sensitive outwardly rectifying (VSOR) anion channels without cell swelling via a mechanism that is regulated by high intracellular Ca^{2+} in nanodomains (159). Although we did not test directly whether this pathway plays a role in bradykinin-induced $[Ca^{2+}]_i$-dependent ATP release from human subcutaneous fibroblasts, it appears that VSOR currents exhibit a slow activation kinetics requiring 15-20 min after bradykinin application to reach a sustained plateau (159). This activation pattern is entirely different from the rapid (within 30 s) ATP releasing response to bradykinin observed in human fibroblasts (see Figure 2.2.3), thus indicating that slow activating but prolonged VSOR currents would play a minor, if some, role in the release of ATP in our experimental time frame. In our hands mefloquine (MFQ, 3 μM, Figure 2.2.4Bi), which also blocks non-selectively anion channels, failed to modify bradykinin-induced $[Ca^{2+}]_i$ signals. Moreover, modulation of VSOR channel

permeability through the activation of protein kinase C with the phorbol ester (12-myristate 13-acetate, 1-10 μM, $n = 3$) did not mimic the effect of bradykinin.

Involvement of soluble signaling mediators, such as ATP and/or its metabolites, may also explain heterogeneity of individual $[Ca^{2+}]_i$ responses to bradykinin within a cell population; confocal microscopy studies showed that some cells displayed no plateau phase whereas others were not noticeably affected by bradykinin removal and continued to respond for a few minutes (see Figure 2.2.1C). This is consistent with the generation of concentration gradients by released ATP and metabolites formation (namely ADP) via ectonucleotidases, which enables differential targeting of subtype-specific P2 purinoceptors and, thus, cell-to-cell communication depending on proximity. Therefore, we may speculate that bradykinin-stimulated fibroblasts trigger a "purinergic wave" mediated by released ATP and metabolites formation that can affect sensory afferent nerve endings localized in the vicinity, representing the first insights of a fibroblast-neuron communication unproved so far.

Recently, it has been reported that mechanical stimulation of human epidermal keratinocytes induces propagating Ca^{2+} waves depending on non-vesicular release of ATP through connexin hemichannels (151). In view of the potential contribution of the cutaneous release of ATP to acute and chronic pain syndromes, this and other groups demonstrated that human epidermal keratinocytes co-cultured with neurons of the dorsal root ganglia interplay through the release of ATP following keratinocytes-born $[Ca^{2+}]_i$ waves (160). Likewise, subcutaneous inflammation or injection of ATP causes pain sensation through the activation of P2X3 receptors expressed in sensory nerve endings, which may become sensitized in both animal models and human patients (161,162). Knocking down or selectively antagonizing P2X3 receptor activity results in reduced responses to ATP, as well as reduced thermal and mechanical hyperalgesia in inflammatory and neuropathic pain rat models (163,164). P2Y purinoceptors, especially $P2Y_1$ and $P2Y_2$, expressed in primary sensory endings have also been implicated in chronic pain states (160). Authors from the latter study agree that cutaneous ATP release does not appear to contribute to pain sensation in the absence of tissue injury. However, under chronic painful conditions, such as inflammation and nerve injury, nerve endings may become sensitized and a normally innocuous level of subcutaneous ATP may now be sufficient to reach the firing threshold of nociceptors (165). Despite direct modulation of nociceptors threshold by ATP released from different cell types

may play a key role to the association between subcutaneous connective tissue injury and musculoskeletal pain, there are alternative mechanisms that should also be considered in this context. These include interference with muscle tension due to fusimotor effects induced by group III and IV afferents activation, which by projecting to γ-motoneurons amplify muscle spindles activity and, thereby, increases muscle tone, generating metabolites (*e.g.* bradykinin) that enter in a positive feedback loop (reviewed by (144,145,166)).

In addition, it has been proposed that mechanical deformation of the skin by needles and application of heat or electrical current leads to release of large amounts of ATP from keratinocytes, fibroblasts and other cells in skin (149). Impulses generated by P2 purinoceptors in sensory fibers in the skin connect with interneurons that may negatively modulate neural pathways to the pain centers in the cortex. This is the basis for the novel hypothesis for the involvement of purinergic signaling in acupuncture (149). Furthermore, purines are known to cause intracellular Ca^{2+}-dependent transient changes in cultured human fibroblast cytoarchitecture, which share similarities with the increase in cross-sectional area of fibroblasts in response to acupuncture (71). Although evidence has been presented of the role of adenosine in acupuncture-mediated anti-nociception by demonstrating that the local concentrations of the nucleoside increase in human subjects (167) and that adenosine is implicated in the proliferation of fibroblasts and remodeling of the skin, liver and lung (reviewed in (66), we failed to demonstrate any contribution of the nucleoside to bradykinin-induced $[Ca^{2+}]_i$ signals in cultured human subcutaneous fibroblasts. Controversy still exists regarding adenosine participation in wound healing and scarring. For instance, the adenosine A_{2A} receptor promotes skin fibrosis and scarring (168) and increases collagen production in human dermal fibroblasts (169) probably by activating the G_s/cyclicAMP pathway (170), yet it remains controversial how A_{2A} receptors increase collagen production since cyclicAMP has been found to decrease the synthesis of collagen and DNA by fibroblasts (171). Thus, further studies are required to test long-term effects of extracellular adenosine, which may originate from the hydrolysis of ATP released from fibroblasts stimulated either mechanically (e.g. acupuncture) or by inflammatory mediators, such as bradykinin. Our findings demonstrating that adenosine accumulates as an end product of the catabolism of released ATP in the vicinity of fibroblasts within the subcutaneous connective tissue may be of clinical relevance, given the role of the nucleoside on dermal fibrosis (via A_{2A} receptors) and its anti-nociceptive

properties (via A_1 receptors) on free nerve endings and sensory afferents (66,172).

CONCLUSIONS

Data indicate that stimulation of constitutively expressed B_2 receptors with bradykinin elicits the opening of hemichannels containing Cx43 and Panx1 subunits, followed by ATP diffusion out of human subcutaneous fibroblasts. ADP originating from the catabolism of released ATP by membrane-bound ectonucleotidases acting in an autocrine or paracrine way on plasma membrane $P2Y_{12}$ purinoceptors may contribute to sustain elevated $[Ca^{2+}]_i$ levels in neighboring cells. Thus, targeting the pathways leading to nucleotides release and the purinergic cascade in human fibroblasts of the subcutaneous tissue may be useful in designing novel therapeutic strategies for tuning the communication between inflammatory cells, fibroblasts and sensory nerve endings, which are key players in the pathogenesis of painful musculoskeletal diseases with widespread involvement of the subcutaneous connective tissue (*e.g.* fibromyalgia).

PAPER 3

(MANUSCRIPT IN PREPARATION)

Adenosine stimulates growth and type I collagen production by fibroblasts of the human subcutaneous tissue via A_{2A} receptors

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ABSTRACT

Changes in connective tissue remodeling are involved in chronic musculoskeletal pain. Adenosine is a potent modulator of inflammation and tissue repair. However, controversy still exists regarding adenosine participation in wound healing and fibrosis (see e.g. (66)). Previously we showed that although adenosine accumulates in the extracellular milieu following the catabolism of released adenine nucleotides, the nucleoside failed to demonstrate any contribution to $[Ca^{2+}]_i$ signals in cultured human subcutaneous fibroblasts challenged acutely with inflammatory mediators (e.g. bradykinin). Here, we analyzed the expression of all four adenosine P1 receptors in human subcutaneous fibroblasts and investigated their role in cell proliferation and type I collagen production during 28 days of cell cultures. Data showed that adenosine accumulation in the cultures may result from reduced adenosine deaminase activity in these cells ($t_{½}$ of 3 μM ADO, 40.1 ± 8.3 min) as compared with the activity of the adenosine forming enzyme, ecto-5'-nucleotidase ($t_{½}$ of 3 μM AMP, 1.5 ± 0.2 min). In addition, human subcutaneous fibroblasts express a significant immureactivity against the A_{2A} receptor; these cells show weak labeling with A_{2B} and A_3 antibodies and did not stain with the A_1 receptor antibody. Prolonged exposure (during 28 days) of human subcutaneous fibroblasts to adenosine or its stable analogues, NECA and CGS 21680, significantly increased fibroblasts growth and type I collagen production. In conclusion, adenosine, via A_{2A} receptors activation, promotes proliferation and type I collagen synthesis by human subcutaneous fibroblasts, which might suggest that these receptors play an active role in connective tissue remodeling and, thus, in chronic musculoskeletal pain.

Keywords: human subcutaneous fibroblasts, adenosine, A_{2A} receptor, proliferation, type I collagen

INTRODUCTION

Subcutaneous connective tissue disorganization appears to occur in patients with chronic pain, possibly as a result of chronic inflammation and fibrosis (13,14,16). Recently, it has been suggested that purinergic signaling may potentially play a role in subcutaneous connective tissue remodeling, namely due to the activation of P2 receptors (70,71,173). There is, however, lack of evidence regarding the purinoceptor subtypes that are expressed in the human subcutaneous connective tissue.

On the other hand, it seems that adenosine, a ubiquitous purine nucleoside and the final active metabolite of ATP, might rapidly appear in the extracellular fluid of human subcutaneous fibroblasts due to the action of membrane-bound ecto-NTPDases that catabolize adenine nucleotides. In fact, we previously demonstrated that AMP is rapidly catabolized into adenosine in cultured human subcutaneous fibroblasts (173), suggesting that adenosine might accumulate and play an important role in subcutaneous connective tissue physiology.

Furthermore, there is increasing evidence that, depending on the tissue, adenosine might play a role in wound healing which, under certain circumstances, may lead to tissue fibrosis (66). In ischemic, hypoxic or inflamed conditions, adenosine levels in the extracellular fluid may be extremely elevated. Nevertheless, tissue adenosine accumulation in response to stressful stimuli might have a dual modulatory role in homeostasis. While adenosine first acts as an alarm molecule that reports injury to the surrounding tissue, it then generates a range of tissue responses that can be generally viewed as organ protective (174). Adenosine effects are mediated by differential activation of four G protein-coupled P1 receptors, namely A_1, A_{2A}, A_{2B} and A_3, each one may undertake a specific role. However, depending on the tissue, evidences have been provided showing that activation of the same receptor may induce opposing effects. For instance, the adenosine A_{2B} receptor inhibits fibrosis in the heart, but it promotes fibrosis in the lung. In the skin, liver, and lungs, both A_{2A} and A_{2B} receptors operate by increasing fibrosis (66).

Given that, to our knowledge, evidence about the role of adenosine in human subcutaneous connective tissue remodeling is missing, we analyzed the expression of all four types of P1 purinoceptors, as well as their role in fibroblasts proliferation and type I collagen production.

EXPERIMENTAL PROCEDURES

Cell cultures

Human fibroblasts were isolated from the subcutaneous tissue of organ donors (45 ± 6 years old (mean ± standard error of the mean), n = 12) with no clinical history of connective tissue disorders. The protocol was approved by the Ethics Committee of Hospital Geral de Santo António SA (University Hospital) and of Instituto de Ciências Biomédicas de Abel Salazar (Medical School) of University of Porto. The investigation conforms to the principles outlined in the Declaration of Helsinki. Subcutaneous tissues were maintained at 4-6ºC in M-400 transplantation solution (4.190 g/100 mL mannitol, 0.205 g/100 mL KH_2PO_4, 0.970 g/100 mL $K_2HPO_4{\cdot}3H_2O$, 0.112 g/100 mL KCl, and 0.084 g/100 mL $NaHCO_3$, pH 7.4) until used, which was between 2 and 16 hours after being harvested (84). Cells were then obtained by the explant technique and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 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 until near confluence (~3-4 weeks), then adherent cells were enzymatically released with 0.04% trypsin-EDTA solution plus 0.025% type I collagenase in phosphate-buffered saline (PBS). The resultant cell suspension was cultured and maintained in the same conditions mentioned above. All the experiments were performed in the first subculture.

Antibody production

Genetic immunization protocol was carried out with pcDNA3.1 plasmid encoding for human CD73 using Hartley guinea pigs, obtained from Charles River Laboratories (Quebec City, Canada).

Immunocytochemistry

Human fibroblasts were seeded in chamber slides at a density of $2.5x10^4$ cells/mL and allowed to grow for 5-15 days. Cultured cells were fixed in 4% paraformaldehyde (PFA) in 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% NaN_3) for 1 h. Primary antibodies, diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X, 0.05% NaN_3), were applied [mouse anti-porcine vimentin 1:75 (DAKO); rabbit anti-human collagen I

1:50 (AbDSerotec); rabbit anti-rat A_1 1:50, rabbit anti-human A_{2B} 1:75 (Chemicon); rabbit anti-canine A_{2A} 1:100 (Alpha Diagnostics); goat anti-human A_3 1:50 (Santa Cruz); and guinea-pig anti-human CD73 1:300 (University of Laval, Québec, Canada; see the Centre de Recherche du CHUL Web site for further details)], and the slides incubated overnight at 4ºC. After incubation, cells were washed 3 times in PBS 1X (10 minutes each). The donkey anti-rabbit IgG Alexa Fluor 488, donkey anti-mouse IgG Alexa Fluor 568, donkey anti-guinea pig IgG Alexa Fluor 568 and donkey anti-goat IgG Alexa Fluor 633 secondary antibodies (Invitrogen) were diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X) and applied for 1h protected from light. A last wash was performed with PBS 1X and glass slides were mounted with VectaShield medium and stored at 4ºC. Observations were performed and analyzed with an Olympus FV1000 confocal microscope (49,93).

Enzymatic kinetic experiments and HPLC analysis

After a 30 minutes equilibration period at 37ºC, cells grown for 11 days were incubated with 3 or 30 μM of adenosine, which was added to the culture medium at zero time. Samples (75 μL) were collected from each well at different times up to 30 minutes for high-performance liquid chromatography (HPLC, LaChrome Elite, Merck, Germany) analysis of the variation of substrate disappearance and product formation (49,89). Adenosine catabolism analysis was performed at room temperature with a LiChrospher 100 RP-18 (5 μm) column (Merck) by isocratic reverse-phase HPLC-UV set at 254 nm (90). The eluent was composed of 91% of 100 mM KH_2PO_4, pH = 7 and 9% of methanol during 10 min with a constant rate flow of 1.25 mL/min. Under these conditions, the retention times of adenosine and its metabolites were as follows: adenosine (8.03 min), inosine (2.73 min) and hypoxantine (1.78 min). Concentrations of the substrate and products were plotted as a function of time (progress curves). The following parameters were analyzed for each progress curve: half-life time ($t_{1/2}$) of the initial substrate, time of appearance of the different concentrations of the products, concentration of the substrate or any product remaining at the end of the experiment. The spontaneous degradation of adenosine at 37ºC in the absence of the cells was (0%) over 30 minutes. At the end of the experiments, the remaining incubation medium was collected and used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity. The negligible activity of LDH in the samples collected at the end of the experiments is an indication of the integrity of the cells during the experimental period.

Cell viability/proliferation

Viability /proliferation studies included the MTT assay as previously described (50). Human fibroblasts were seeded in flat bottom 96 well plates at a density of $3x10^4$ cells/mL and cultured in supplemented DMEM as described before. Cell cultures were routinely monitored by phase contrast microscopy and characterized at days 1, 7, 14, 21 and 28. The 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 4 h of each test period, cells were incubated with 0.5 mg/mL of MTT for 4 h 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 (Synergy HT, BioTek). Results were expressed as A/well.

Total type I collagen determination

Type I collagen determination was performed using the Sirius Red staining assay. Human fibroblasts were cultured as described for the viability/proliferation studies. The staining protocol was adapted from Tullberg-Reinert & Jundt (1999) (88). Cell layers were washed twice in PBS before fixation with Bouin's fluid for 1 h. The fixation fluid was removed by suction and the culture plates were washed by immersion in running tap water for 15 minutes. Culture dishes were allowed to air dry before adding the Sirius Red dye (Direct Red 80). Cells were stained for 1 h under mild shaking on a microplate shaker. To remove non-bound dye, stained cells were washed with 0.01 N hydrochloric acid and then dissolved in 0.1 N sodium hydroxide for 30 minutes at room temperature using a microplate shaker. Optical density was measured at 550 nm against 0.1 N sodium hydroxide as blank (88). Results were expressed as A/well.

Materials and reagents

Amphotericin B, bovine serum albumin (BSA), Dulbecco's Modified Eagle's Medium (DMEM), ethylene diaminetetraacetic acid (EDTA), fetal bovine serum (FBS), 5′-(N-ethylcarboxamide)adenosine (NECA), penicillin/streptomycin, phosphate buffered saline system (PBS), and type I collagenase were purchased from Sigma-Aldrich. 2-(2-Furanyl)-7-[3-(4-methoxyphenyl-)propyl]-7H-pyrazolo[4,3-

e][1,2,4]triazolo[1,5-c]p-yrimidin-5-amine (SCH 442416), and 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofu-ranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzene-propanoic acid hydrochloride (CGS 21680) were obtained from Tocris Cookson Inc.. Dimethylsulphoxide (DMSO) and glacial acetic acid were obtained from Merck and Bouin's solution was acquired from Panreac. CGS 21680, NECA, and SCH 442416 were prepared in dimethyl sulfoxide (DMSO). All other drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of DMSO at the maximal concentration used (0.05% v/v), were observed. The pH of the solutions did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

Tissue culture plates: 96-well plates were purchased from Corning; chamber slides were from Nunc.

Presentation of data and statistical analysis

Data are expressed as mean ± S.E.M. from an n number of experiments/cells/individuals. Data from different individuals were evaluated one-way analysis of variance (ANOVA). Statistical differences found between control and drug-treated cultures were determined by the Bonferroni's method. p values < 0.05 were considered to represent significant differences.

RESULTS

Extracellular catabolism of adenosine by human subcutaneous fibroblasts in culture

Cell cultures obtained from subcutaneous connective tissue exhibited a positive immunoreactivity against the fibroblast-cell markers, vimentin and type I collagen (Fig. 2.3.1A). Human subcutaneous fibroblasts also exhibited strong immunoreactivity against the ecto-5'-nucleotidase (CD73) (Fig. 2.3.1B), the enzyme that is responsible for extracellular AMP dephosphorylation, thus enabling fast adenosine formation from released adenine nucleotides. Analyzing the kinetics of the extracellular catabolism of exogenously applied adenosine, one may conclude that adenosine deamination occurs slowly in cultured human subcutaneous fibroblasts, which allows adenosine to accumulate in the

extracellular milieu (Fig. 2.3.1C). When cells were incubated with 3 μM adenosine, inosine was the only metabolite detected whose concentration slowly increased with time, reaching the maximum value of 0.96 ± 0.58 μM after 30 min of adenosine application (Fig. 2.3.1Ci). Adenosine (3 μM) was metabolized with a half-life time ($t_{1/2}$) of 40.1 ± 8.3 min (*n* = 4 observations from 2 individuals). Increasing the concentration of the substrate to 30 μM adenosine, formation of inosine increased to the maximum value of 4.05 ± 2.83 μM after 30 min of incubation; formation of hypoxanthine was negligible (less than 1 μM) (Fig. 2.3.1Cii). Adenosine (30 μM) was metabolized even more slowly; a $t_{1/2}$ of 157.6 ± 16.6 μM was calculated (*n* = 4 observations from 2 individuals). The semi-logarithmic representation of progress curves obtained by polynomial fitting of the nucleoside catabolism show a linear pattern ($y = -0.0051x + 0.4578$ and $R^2 = 0.99$ for 3 μM: $y = -0.0015x + 1.4640$ and $R^2 = 0.99$ for 30 μM). Given that the slope of the progress curves decreased upon increasing the concentration of the substrate from 3 to 30 μM, data indicate that the kinetics of the catabolism of adenosine is more slowly whenever the concentration of the nucleoside increases, thus favoring its extracellular accumulation.

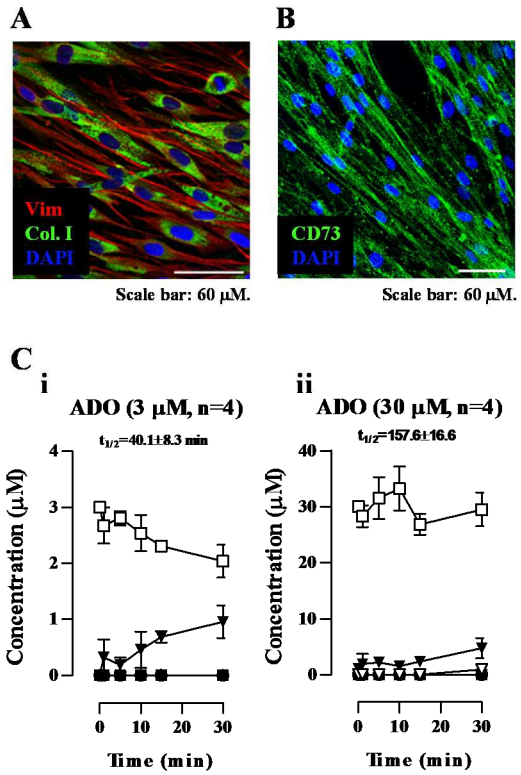

Figure 2.3.1. Adenosine accumulation in human subcutaneous fibroblasts in culture. Panel **A** demonstrates the immunoreactivity against vimentin (red), which has been described as a reliable fibroblast marker, as well as the staining of type I collagen (green), which is produced by activated fibroblasts. Panel **B** shows immunoreactivity of human subcutaneous fibroblasts against ecto-5'-nucleotidase (CD73). DAPI (blue) represents the staining of cell nucleus; images were obtained under confocal microscope and are representative of, at least, three individuals; scale bar is 60 µm (**A** and **B**). Panel **C** shows the time course of the extracellular catabolism of adenosine (3 µM, **i**; and 30 µM, **ii**) in human subcutaneous fibroblast cultures grown during 11 days. Adenosine was added to the culture medium at time zero. Samples (75 µl) were collected from each well at indicated times in the abscissa. Each collected sample was analyzed by HPLC to separate and quantify AMP (filled squares), adenosine (open squares), inosine (filled triangles) and hypoxanthine (open triangles). Each point represents pooled data from two individuals; 2 replicas were performed in each individual experiment. Vertical bars represent S.E.M. and are shown when they exceed the symbols in size. The calculated half-life time ($t_{1/2}$, min) for each initial substrate is shown for comparison.

P1 receptor expression in human subcutaneous fibroblasts in culture

The expression of P1 receptors in human subcutaneous fibroblasts in culture was assessed by immunofluorescence confocal microscopy using cells from, at least, 3 individuals. Results provided clear evidence for the expression of A_{2A} receptors in these cells (Fig. 2.3.2). In contrast, human subcutaneous fibroblasts showed weak positive immunoreactivity against the A_{2B} and A_3 receptors and no staining was detected for the A_1 receptor (Fig. 2.3.2). Omission of primary antibodies from the incubation buffer completely prevented secondary antibody labeling (data not shown).

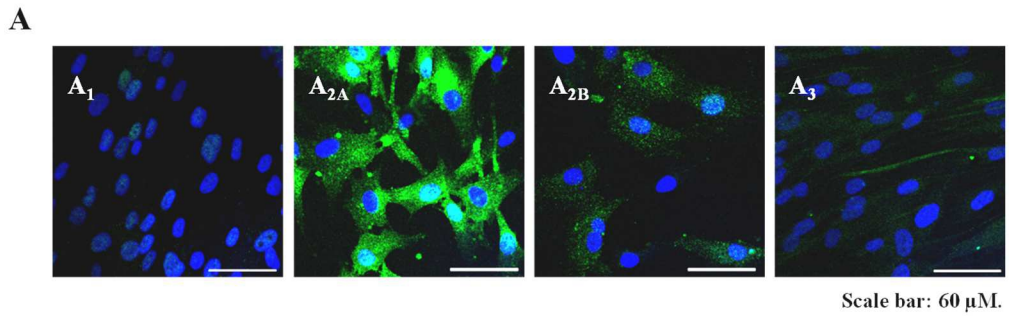

Figure 2.3.2. Immunocytochemical staining of adenosine P1 receptors in fibroblasts isolated from human subcutaneous tissue. Panel shows immunoreactivity of human subcutaneous fibroblasts against A_1, A_{2A}, A_{2B} and A_3 receptors; cells grown for 7 days. Images obtained under confocal microscope; representative of, at least, three individuals. Scale bar is 60 µm.

Effect of adenosine on proliferation/viability and type I collagen production by human subcutaneous fibroblasts

To investigate the involvement of adenosine P1 receptors on cell proliferation/viability (through the MTT method) and extracellular matrix production, namely type I collagen production (Sirius Red assay), fibroblasts were continuously incubated (during 28 days) with adenosine (100 µM) or its stable analogue 5′-(N-ethylcarboxamide)adenosine (NECA, 100 µM). Given that the immunocytochemical analysis showed a strong immunoreactivity of fibroblasts against the A_{2A} receptor compared to A_1, A_{2B} and A_3 receptors, we also tested the cells with the selective A_{2A} receptor agonist, CGS 21680 (10 nM), with and without the selective antagonist of the A_{2A} receptor, SCH 442416 (10 nM). For each individual, 4-8 replicas were performed. Results obtained showed that human subcutaneous fibroblasts in culture under control conditions (no added drugs) proliferate (Fig. 2.3.3A) and produce type I collagen (Fig. 2.3.3C) progressively with time in culture. Prolonged exposure to adenosine (100 µM, n = 4) and NECA (100 µM, n = 4) stimulated cell growth (Fig. 2.3.3Bi) and type I collagen production (Fig. 2.3.3Di) above the control condition from day 14 onwards. Human fibroblasts proliferation (Fig. 2.3.3Bi) and type I collagen production (Fig. 2.3.3Di) were more significant ($p < 0.05$) upon the incubation of the cells with the selective A_{2A} receptor agonist, CGS 21680 (10 nM, 4-8 replicas from two individuals). Similarly, the A_{2A} receptor agonist progressively increased ($p < 0.05$) type I collagen production until culture day 28 (Fig. 2.3.3Di). Figures 2.3.3Bii and 2.3.3Dii, show that the effects of CGS 21680 (10 nM) on fibroblast cells growth

and type I collagen production were both attenuated in the presence of the selective A_{2A} receptor antagonist, SCH 442416 (10 nM).

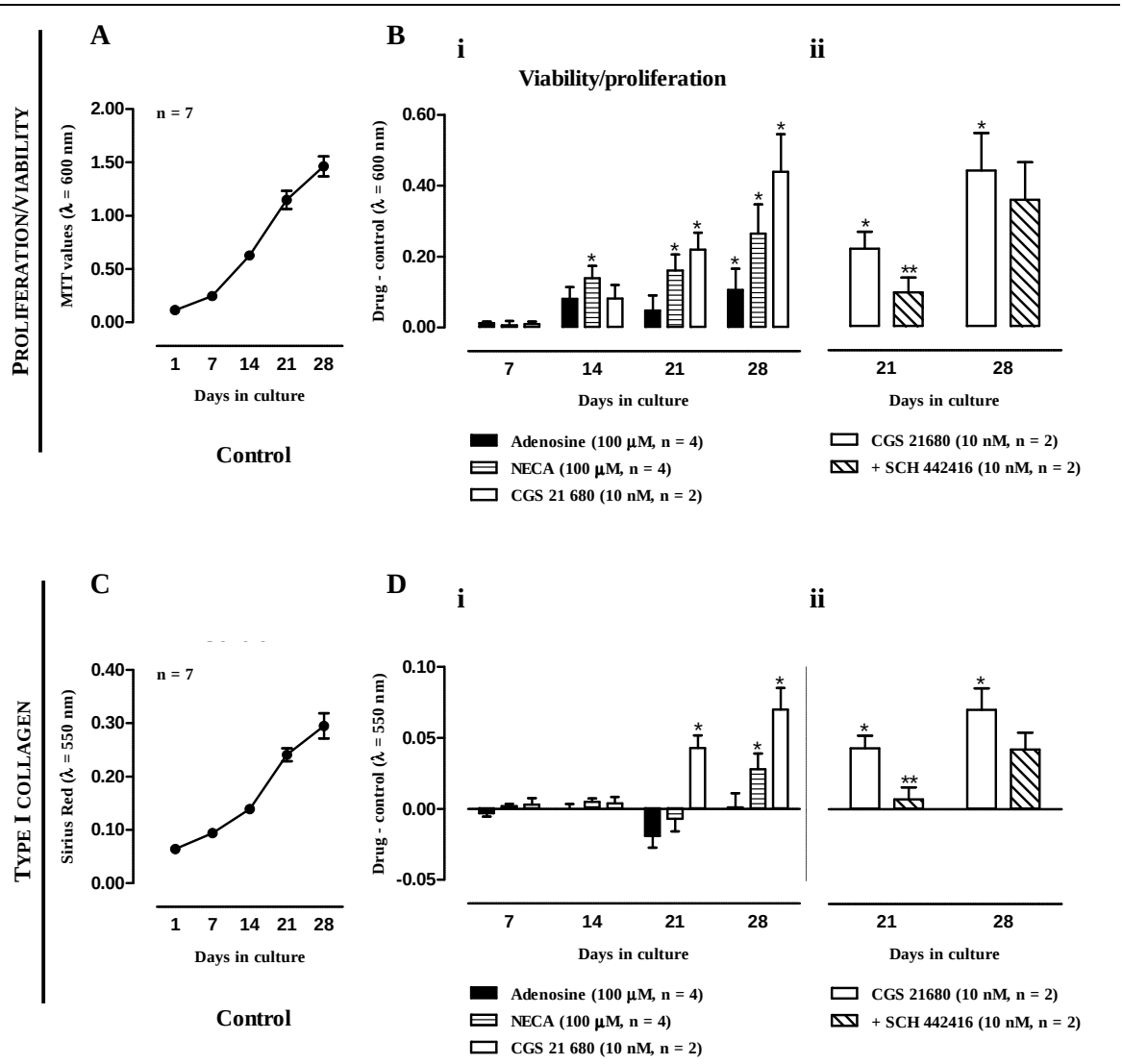

Figure 2.3.3. Proliferation/viability and type I collagen production by human subcutaneous fibroblasts grown for 28 days in culture: role of adenosine. Panels **A** and **B** represent proliferation/viability of cells, measured by the MTT assay; results are expressed as absorbance determination at 600 nm per well at certain time points. Panels **C** and **D** represent total type I collagen production assessed by Sirius Red staining; results are expressed as absorbance determination at 550 nm per well at certain time points. **A** and **C,** illustrate respectively curves of human subcutaneous fibroblasts proliferation/viability (MTT values) and type I collagen production (Sirius Red values). Adenosine (100 μM), the stable non-selective agonist of P1 receptors NECA (100 μM) and the selective agonist of the A_{2A} receptor CGS 21680 (10 nM) were all applied continuously to the culture media; CGS 21680 (10 nM) was applied in the absence and in the presence of the selective A_{2A} receptor antagonist, SCH 442416 (10 nM). The ordinates represent changes in cell growth (MTT values, **Bi** and **Bii**) and type I collagen production (Sirius Red values, **Di** and **Dii**) compared to their respective internal controls (in the absence of test drugs) at the same time points (Panels **B** and **D**). Zero represents similarity between the two values (drug - control); positive and negative values represent facilitation or inhibition of either cell growth or type I collagen production relative to control data obtained at the same time points. Each column represents pooled data *n* individuals; 4-8 replicas were performed for each individual. The vertical bars represent S.E.M.. $*p <$ 0.05 represent significant differences from control values obtained in the absence of tested drugs.

Given the similarity between both proliferation/viability and type I collagen patterns, we normalized collagen production (Sirius Red values) to proliferation/viability (MTT values), at the same time points. Interestingly, after this normalization differences in collagen production were no longer observed for NECA (100 μM, n = 4; Fig. 2.3.4Ai). In the case of adenosine (100 μM, n = 4; Fig. 2.3.4Ai) and CGS 21680 (10 nM, n = 2; Fig. 2.3.4Ai), normalization revealed that type I collagen production increased significantly ($p < 0.05$) beyond proliferation / viability of the human subcutaneous fibroblasts on culture day 28. Moreover, the effect of CGS 21680 (10 nM) was attenuated in the presence of the selective A_{2A} receptor antagonist, SCH 442416 (10 nM).

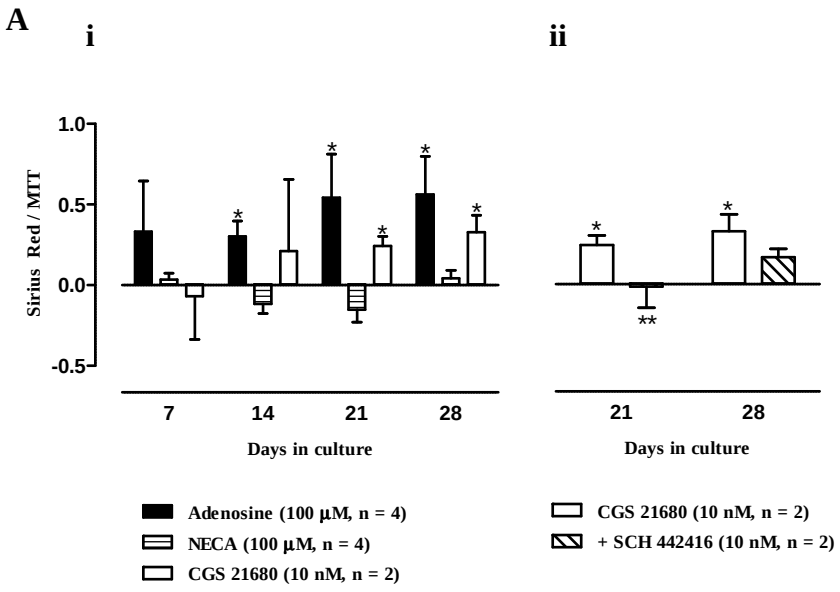

Figure 2.3.4. Type I collagen production normalized to proliferation/viability of human subcutaneous fibroblasts grown for 28 days in culture: role of adenosine. Panel **A** represents type I collagen production, assessed by Sirius Red staining, normalized to the proliferation/viability of cells, measured by the MTT assay; results are expressed as absorbance determination at 550 nm / absorbance determination at 600 nm, per well at certain time points. Adenosine (100 μM, **Ai**), the stable non-selective agonist of P1 receptors NECA (100 μM, **Ai**) and the selective agonist of the A_{2A} receptor CGS 21680 (10 nM, **Ai**), were all applied continuously to the culture media; CGS 21680 (10 nM) was also applied in the presence of the selective A_{2A} receptor antagonist, SCH 442416 (10 nM, **Aii**). Each column represents pooled data n individuals; 4-8 replicas were performed for each individual. The vertical bars represent S.E.M.. *$p < 0.05$ represent significant differences from control values obtained in the absence of tested drugs.

DISCUSSION

Adenosine is present in most biological fluids but its concentration increases during tissue or organ stress, when it acts as an endogenous modulator of inflammation and tissue repair. Under basal conditions, the extracellular adenosine concentration is relatively constant (30-300 nM) and above its intracellular concentration, mostly due to the high affinity of intracellular adenosine kinase for the nucleoside. The extracellular concentration of adenosine may increase dramatically to the micromolar range upon increasing the release of adenine nucleotides, namely ATP, from stressed and/or damaged cells (reviewed by (170)). However, the levels achieved by adenosine in close proximity of its receptors are strictly regulated by a variety of dynamic mechanisms, of which the catabolic enzyme adenosine deaminase (ADA) represents a critical regulator of receptor stimulation, thus playing a pivotal role in the modulation of the purinergic responses (73). Interestingly, it has been shown that ADA knockout mice, which have a systemic elevation of adenosine levels, exhibit signs of fibrosis in the lungs, liver and kidneys, suggesting a profibrotic trend in multiple tissues (74). Still, the role of adenosine in tissue remodeling seems controversial. Though evidence suggests that adenosine promotes fibrosis in several organs through different mechanisms, which might be associated to distinct patterns of adenosine receptors expression in different organs commanding the overall role of the nucleoside (175), it has been described that the same subtype of adenosine receptor (e.g. A_{2B}) may inhibit or promote fibrosis depending on the target tissue (66). Given that subcutaneous connective tissue disorganization appears to be involved in the pathogenesis of chronic pain (13,14,16), we sought it was important to unveil the role of adenosine in human subcutaneous connective tissue remodeling. Despite adenosine receptors have been identified in human dermal fibroblasts, to our knowledge, no previous evidence exists describing the expression of adenosine receptors in the related human subcutaneous (hypodermal) fibroblasts. We have previously demonstrated that adenosine increases following the incubation of cultured human subcutaneous fibroblasts with adenine nucleotides, namely ATP, ADP and AMP (173,176). Now, we confirmed that non-stimulated human subcutaneous fibroblasts exhibit a minor activity of ADA, allowing for adenosine accumulation. Our data also show that the A_{2A} receptor appears to be the most expressed P1 receptor subtype in human subcutaneous fibroblasts in culture. Interestingly, it has been previously shown that the A_{2A} receptor is also the dominant adenosine receptor in the skin, as well

as in the peritoneum and the liver (66), which makes targeting this receptor subtype highly appealing to control tissue remodeling.

To characterize further the contribution of adenosine and the role of A_{2A} receptors in proliferation and collagen production by fibroblasts of the human subcutaneous connective tissue, we tested the effect of adenosine and of two stable analogues with affinity to A_2 receptors, both A_{2A} (CGS 21680C) and A_{2B} (NECA). Results are in agreement with the hypothesis that prolonged exposure to adenosine stimulates human subcutaneous fibroblasts growth and type I collagen production through the activation of the A_{2A} receptor. This was suggested because CGS 21680 was the more potent and effective agonist tested and by the fact that the selective A_{2A} receptor antagonist, SCH 442416 (10 nM), attenuated the effects of CGS 21680. Data is in agreement with the idea that the A_{2A} receptor might be a key player in subcutaneous connective tissue remodeling, and therefore might participate in wound healing disorders, such as fibrosis. In the skin, it has been previously reported that adenosine, acting via the A_{2A} receptor, promotes wound healing and excisional wound closure, and that the enhancement in dermal wound healing is accompanied by an increase of the matrix (collagen) in the wounds. In addition, the A_{2A} receptor plays an important role in the pathogenesis of fibrotic malignancies of the skin such as dermal fibrosis. The blockade of the A_{2A} receptor prevents dermal fibrosis and scarring by reducing collagen content and misalignment (reviewed in (170,175)). Interestingly, we found that adenosine potentiates the ability of each fibroblast to synthetize collagen, similarly to that occurring in the skin where the A_{2A} receptor activation directly stimulates collagen production by dermal fibroblasts (reviewed in (170)).

Previously, it has been described that adenosine can regulate cells proliferation and differentiation possibly due to changes in mitogen-activated protein kinases (MAPK). Among the MAPK family, which is subdivided into extracellular regulated kinases (ERK), like ERK1/2, and stress-activated protein kinases (SAPK), like p38 and jun-N-terminal kinase (JNK), the ERK1/2 seems to represent the preferential pathway for cell division and proliferation responses. These kinases, commonly activated via receptor tyrosine kinases, have also been shown to be activated by G protein-coupled receptors, such as P1 receptors (177). As an example, adenosine analogues exerting mitogenic effects on human endothelial cells via the A_{2A} receptor seem to activate ERK1/2 using the cAMP-ras-MEK1 pathway (177). Therefore, one may speculate that human fibroblasts from

subcutaneous connective tissue might act similarly. However, the signaling cascades used by the A_{2A} receptor seem to vary with the cellular background and the signaling machinery that the cell possesses. For instance, the A_{2A} receptor-mediated ERK1/2 activation in CHO cells is dependent on G_s-cAMP-PKA-rap1-p68 B-raf-MEK1. On the other hand, the A_{2A} receptor-mediated activation in HEK 293 cells involves PKC, ras, and sos, but not G_s, cAMP, or PKA, even though cAMP levels do rise in a G_s-dependent manner (177). Thus, further studies are required to ascertain the transduction pathways involved in the observed A_{2A} receptor-mediated increase of proliferation and type I collagen synthesis by human subcutaneous fibroblasts.

Overall, these data enlighten the idea that the A_{2A} receptor might be a key player in subcutaneous connective tissue remodeling by modulating its elastic properties. Therefore, we hypothesize that a disturbed adenosine homeostasis promotes a deficient remodeling of connective tissue and, given that subcutaneous connective tissue forms an interconnected net throughout all body (7,78), any local increased tension can elicit prolonged release of adenine nucleotides which might induce and sustain changes of all the connective tissue net, further restraining its compliance globally. This occurrence could, eventually, be the basis of a peripheral modulation of the inputs, namely of nociception transmission from peripheral soft tissues, and be involved in the pathogenesis of chronic pain conditions. In fibromyalgia, though a central modulation of the pain circuits has been described, its etiology and pathophysiology has not been clearly understood and it is still unknown if there is a peripheral modulation leading to a central sensitization. Interestingly, recent data indicates that adenosine and inosine may be involved in pain transmission in fibromyalgia patients, who showed an increased activity of serum-ADA (178). This finding suggests that these patients have reduced adenosine levels, which, given that adenosine is known as an important anti-nociceptive mediator, would explain a reduced threshold for pain eliciting. In addition to this, given that under normal physiologic conditions adenosine appears to accumulate in the subcutaneous connective tissue and potentiate fibroblasts growth, which are the major key players in the extracellular matrix remodeling, both by synthetizing new proteins as well as by degrading them, reduced levels of adenosine may restrain the ability of fibroblasts for connective tissue remodeling by limiting their growth. In other words, we postulate that lower levels of adenosine may reduce the regenerative ability of subcutaneous connective tissue due to a decrease of new fibroblasts

growth, leading to viscoelastic changes that might compromise the global compliance of this tissue and, subsequently, reduce the pain thresholds. Thus, we believe that the role of connective tissue in nociception might be complex and purines should be taken into consideration.

In conclusion, adenosine, via the A_{2A} receptor activation, promotes the proliferation and type I collagen production from human subcutaneous fibroblasts, which might suggest that these receptors play an active role in connective tissue remodeling and, thus, in chronic musculoskeletal pain.

CHAPTER 3. DISCUSSION AND CONCLUSIONS

Purines and pyrimidines are involved in the fine-tuning modulation of inflammatory events and painful conditions, such as musculoskeletal pain-related disorders (109,179). There is, however, considerable speculation about the mechanism(s) by which purinergic signals operate musculoskeletal pain. In patients with chronic low back pain, signs of peripheral soft tissue changes have been found, namely subcutaneous connective tissue disarrangement (e.g. increased stiffness, thickness or disorganization) (14,16) suggesting that connective tissue might be an important player in the pathogenesis of chronic musculoskeletal pain. Interestingly, the analgesic effects induced by acupuncture seem to involve the release of ATP and/or its metabolites, including adenosine (142,167). Given that acupuncture is typically applied to deep tissue (142,167), it is more likely that deeper structures than the skin, such as the subcutaneous connective tissue, will play a pivotal role in mediating the effects of acupuncture. Altogether, these evidences point out that subcutaneous connective tissue might represent an active structure that influences afferent nociceptive circuitries from the periphery, modulating pain.

In order to understand the underlying purinergic mechanisms that could be involved in connective tissue remodeling under stressful and non-stressful conditions, we decided to isolate its major cell constituent, fibroblast. Given that: 1) histamine and bradykinin are released or synthesized, respectively, following tissue injury which can occur for example upon sustained muscle contraction; and that 2) these inflammatory mediators have been shown to induce the release of ATP in several cell types, such as smooth muscle fibers, epithelial, endothelial and urothelial cells, as well as various immortalized cell lines, we investigated the involvement of ATP in histamine- and bradykinin-induced response in human subcutaneous fibroblasts in culture.

First of all, our results provided evidence that human subcutaneous fibroblasts are sensitive to both histamine (100 μM) and bradykinin (> 1 μM), showing an increase of the cytosolic intracellular Ca^{2+} ($[Ca^{2+}]_i$) via the activation of H_1 and B_2 receptors, respectively. The histamine- and bradykinin-induced $[Ca^{2+}]_i$ rises depended on PLC activation and recruitment of Ca^{2+} from intracellular stores, which is compatible with the involvement of the $G_q \rightarrow PLC \rightarrow IP_3$ signaling pathway that is characteristic of H_1 and B_2 receptors activation. Interestingly, for both inflammatory mediators the $[Ca^{2+}]_i$ responses produced by the cells were biphasic, showing a fast Ca^{2+} rise component which declined within 1 min to a sustained plateau above baseline, which was maintained via Ca^{2+} entry from the extracellular

milieu. Confirming our initial hypothesis, we found that the observed sustained $[Ca^{2+}]_i$ elevation was partially due to P2 purinoceptors activation. In parallel to the observed $[Ca^{2+}]_i$ oscillations, ATP release in response to histamine and bradykinin was also detected, further corroborating purines involvement in the inflammatory response of these mediators.

Notably, both the nucleotide-releasing pathways triggered by each inflammatory mediator and the subsequent activated P2 purinoceptor subtypes seem to be different. While both histamine and bradykinin caused the opening of pannexin-1 hemichannels, involvement of connexin hemichannels (possibly Cx43 and others) seems to be involved only in bradykinin-induced responses. This finding suggests that, although both H_1 and B_2 receptors couple to a G_q protein, distinct downstream pathways might be stimulated. Response variability may arise from the fact that receptors engage different transducing elements and PLC isoforms in a microdomain-specific manner, like lipid rafts or caveolae, which are characterized by bringing together receptors, G proteins and effector systems. For instance, it has been described that Cx43 interacts with caveolins (caveolin-1, Cav-1) and targets to lipid rafts domains (180). Moreover, it has been proposed that the association between Cav-1 and Cx43 may work like a molecular chaperone or scaffold (181). There is broad evidence that association of transmembrane receptors and signaling molecules within lipid rafts/caveolae provides an enriched environment for protein-protein interactions necessary for signal transduction. Several receptors translocate into submembrane compartments after ligand binding, while others move in the opposite direction (182). The role of such a dynamic localization and functional relevance in signaling modulation and receptor desensitization or internalization requires further investigation in the target cells used in this study, the fibroblasts of the human subcutaneous tissue. Moreover, disruption of submembrane compartments can shift the purinergic receptors from raft/caveolar to non-raft/non-caveolar fractions, and then abolish their ability to activate lipid signaling pathways and to integrate with additional lipid-controlled signaling events. This might modulate the biological response to purinergic ligands and, above all, indicates that the topology of the various purinergic components at the cell surface not only organizes the signal transduction machinery, but also controls the final cellular response (182). This ability of rapid rearrangement of the cell machinery might also explain the fact that histamine-evoked response involved $P2Y_1$ receptor, whereas bradykinin elicited $P2Y_{12}$ receptor activation.

Interestingly, lipid rafts are required in $G\alpha_i$ signaling downstream of the $P2Y_{12}$ receptor during ADP-mediated platelet activation (183). Together, these facts may help to understand that, in the same cell, histamine- and bradykinin-induced purinergic potentiation loops might involve distinct, complex and dynamic microdomains, whose organization deserves further research.

Interestingly, in both histamine- and bradykinin-induced purinergic signaling, ATP catabolism into ADP was required. Although E-NTPDase1 was found to be the most representative E-NTPDase expressed by human subcutaneous fibroblasts, transient ADP accumulation might be possible given that expressed E-NTPDase2 is a preferential nucleoside triphosphatase that hydrolyses ATP 10 to 15 times more efficiently than ADP (39,184). Remarkably, we also found that AMP was rapidly dephosphorylated into adenosine independently of the nucleotide substrate used (half-life time of 1.5-3 min, 3-30 μM, respectively). A strong immunoreactivity against ecto-5'nucleotidase/CD73 corroborated the functional HPLC analysis, suggesting that adenosine might play an important role in the physiology of fibroblasts and, ultimately, connective tissue remodeling. Although we failed to demonstrate any contribution of the nucleoside to bradykinin-induced $[Ca^{2+}]_i$ signals, our results suggest that adenosine, possibly via A_{2A} receptor activation, potentiates human subcutaneous fibroblasts growth and type I collagen production. Interestingly, adenosine receptors have been associated with controversial roles, like wound healing and tissue fibrosis. For instance, in the heart, the A_{2B} receptor seems to inhibit fibrosis, whereas in the lung promotes it. On the other hand, in the skin, liver, and lungs, both A_{2A} and A_{2B} receptors mediate and increase fibrosis (66). In fact, it was demonstrated that adenosine A_{2A} receptors promote skin fibrosis and scarring (66,168) by increasing collagen production in human dermal fibroblasts (169) probably via the G_s/cAMP pathway (170), though cAMP also may decrease collagen production (171). These findings demonstrate that the role of adenosine in these processes is still unclear. Therefore, and despite the generation of adenosine from the catabolism of the released nucleotides does not seem to contribute to the acute bradykinin exposure-induced response in human subcutaneous fibroblasts, further studies are required to better understand which situations might trigger adenosine signaling. Moreover, given that adenosine accumulates as an end product of the catabolism of released ATP in the vicinity of fibroblasts within subcutaneous connective tissue, neighboring structures, such

as free nerve endings, may become a potential target to the well-known anti-nociceptive nucleoside via A_1 receptor activation (172).

Besides adenosine, ATP and ADP may also evoke a paracrine response to the neighboring cells. In the intestine, it has been demonstrated that the ATP released by mechanically stimulated fibroblasts elicits a Ca^{2+}-wave propagation through the fibroblasts' network via $P2Y_1$ receptor activation. The released ATP, acting on P2X receptors localized in the terminals of mucosal sensory neurons, also seems to regulate the intestinal peristaltic motility (57). Likewise, it has been reported that human epidermal keratinocytes co-cultured with neurons from DRG interplay through the release of ATP following keratinocytes-born $[Ca^{2+}]_i$ waves, strongly indicating a dynamic cross-talk between both cell types via extracellular ATP (151,160).

Therefore, we may speculate that histamine- and bradykinin-stimulated fibroblasts trigger a "purinergic wave" operated by ATP release and formation of its metabolites in the extracellular milieu, which can affect sensory nerve afferents localized in the vicinity, representing the first insights of a subcutaneous fibroblast-neuron communication unproved so far. In fact, subcutaneous inflammation or injection of ATP causes pain sensation through the activation of P2X3 receptors expressed in sensory nerve endings, which may become sensitized in both animal models and human patients (161,162). Knocking down or selectively antagonizing P2X3 receptor activity results in reduced responses to ATP, as well as reduced thermal and mechanical hyperalgesia in inflammatory and neuropathic pain models in the rat (163,164). P2Y purinoceptors, especially $P2Y_1$ and $P2Y_2$, expressed in primary sensory endings have also been implicated in chronic pain situations (160). Several authors agree that cutaneous ATP release does not appear to contribute to pain sensation in the absence of tissue injury. However, under chronic painful conditions, such as inflammation and nerve injury, nerve endings may become sensitized and a normally innocuous level of subcutaneous ATP may then be sufficient to reach the firing threshold of nociceptors (165). Despite the fact that direct modulation of nociceptors threshold by ATP released from different cell types may play a key role to the association between subcutaneous connective tissue injury and musculoskeletal pain, there are alternative mechanisms that should also be considered in this context. These include interference with muscle tension due to fusimotor effects induced by group III and IV afferents activation, which, by projecting to γ-motoneurons, amplify muscle spindles activity and,

thereby, increase muscle tone, generating metabolites (*e.g.* bradykinin) that enter in a positive feedback loop (reviewed by (144,145,166)). Moreover, such a motor control dysfunction, which represents a sub-optimal neuromuscular control, also leads to a decreased ability to provide the needed articular stability of the mobilizing segments, therefore contributing to a mechanical instability, thus promoting more inflammation and pain.

It has been proposed that mechanical deformation of the skin by acupuncture needles and application of heat or electrical current leads to the release of large amounts of ATP (and adenosine) from keratinocytes, fibroblasts and other cells in the skin (149). According to this hypothesis, the released ATP generates P2 purinoceptors-dependent impulses in sensory fibers of the skin, which connect with interneurons that may negatively modulate neural pathways to the pain centers in the cortex. This is the basis of the novel hypothesis for the involvement of purinergic signaling in acupuncture (149). However, and though the skin is penetrated during such procedures, acupuncture is typically applied to deeper tissues, like muscles and connective tissue (142,167). Therefore, as mentioned above, it is more likely that deeper structures, such as the subcutaneous connective tissue, deep fascia and muscles, are the key players mediating the anti-nociceptive effects of acupuncture.

Recent studies provided important findings about purines mediating the analgesic effects of acupuncture (142,167). In mice, acupuncture locally increased the extracellular concentrations of ATP, ADP, AMP and adenosine. The authors also observed that the extracellular concentration of ATP returned to baseline after acupuncture, whereas adenosine, AMP and ADP remained significantly elevated for at least more 60 min. Further experiments pointed out that adenosine, via A_1 receptor activation, might be mediating the acute analgesic effects of acupuncture. Using sections of tissue (skeletal muscles with the overlying subcutaneous tissue), the authors found out that AMP dephosphorylation seems to be the rate limiting step in adenosine production (142). Consistently with other reports (89,185), Goldman and coworkers (142) found that IMP was generated in much larger quantities than adenosine due to AMP deaminase, which functions as an enzymatic shuttle for AMP catabolism that bypasses adenosine production (89). Moreover, the authors found that deoxycoformycin, which inhibits AMP deaminase and adenosine deaminase, thereby suppressing the two major pathways involved in the elimination of extracellular adenosine, significantly increased the accumulation of adenosine

and suppressed IMP and inosine production in the isolated preparations (142). Interestingly, our results show that in subcutaneous fibroblasts maintained in culture, AMP is rapidly dephosphorylated into adenosine, yet no formation of IMP was detected, thus promoting the accumulation of the nucleoside. Therefore, we speculate that the major evidence seen by Goldman and coworkers (142) might be attributable to the muscular component of their preparation, where IMP and inosine formation is much more representative than in the connective tissue (89). These findings clearly indicate that purinergic signaling is different between muscle fibers and fibroblasts, and suggest that connective tissue might be the major key player in mediating the rapid adenosine formation seen after acupuncture. Similarly to acupuncture, ultrasound and other manipulative treatments that involve the mechanical challenge of the connective tissue, might also be associated with an efflux of cytosolic ATP that is sufficient to elevate extracellular adenosine and dampen pain in part through the activation of A_1 receptor in the sensory afferents of ascending nerve fibers.

Besides the referred direct and indirect influence on nociception circuitries, the tension of the connective tissue might also influence the sensibility of the surrounding cells to trigger the release of nucleotides release to the extracellular space. Given that mechanical stimulation is known to evoke ATP release in several cells, in cases of increased mechanical tension due to changes in the viscoelastic properties of the connective tissue, for example due to fibrosis, a minor stimulus might be sufficient to induce ATP release by the neighboring cells of free nerve endings, thus modulating their activity. In addition, an efficient muscle contraction requires an optimal muscle fiber length, which is also influenced by the tension of the surrounding connective tissue, within which muscle fibers are inserted into. Thus, the role of connective tissue in nociception might be much more complex that it might look like.

Concluding, human subcutaneous fibroblasts express several purinergic receptors, both P2 and P1 receptors. Under stressful situations, such as via the exposure to inflammatory mediators like histamine and bradykinin, human subcutaneous fibroblasts release ATP to the extracellular milieu, which concur to amplify the response induced by the primary ligand. For each mediator, the underlying signaling pathways might depend on specific microdomains within the cells, triggering a stimulus-specific rather than a cell-specific response. This signature involves different nucleotide-releasing pathways (Panx1 and Cx43 hemichannels, among other connexins) and the activation of distinct purinoceptor

subtypes ($P2Y_1$ and $P2Y_{12}$), which might be determined by a dynamic mobilization of ectonucleotidases (probably E-NTPDase2) to specific regions of the cell membrane. Although we speculate that other stimuli, like mechanical, might trigger a purinergic signaling using the cellular machinery described above, we cannot rule out the involvement of other purinergic elements. Moreover, the fact that, in these cells, adenosine tends to accumulate in the extracellular fluid and that, via A_{2A} receptor activation, adenosine seems to increase proliferation and type I collagen production, suggest that conditions that trigger huge amounts of ATP release, like cell damage, may involve other purinergic signaling units.

Thus, targeting the pathways leading to nucleotides release and subsequent purinergic signaling in human fibroblasts of subcutaneous connective tissue may be useful in: 1) understanding the role of this tissue in the pathogenesis of chronic painful musculoskeletal disorders; 2) clarifying the mechanisms that may be involved in the analgesic effects induced by peripheral soft tissue manipulations, such as physical therapy techniques; and 3) enlightening for novel pharmacological therapeutic strategies towards the modulation of the communication between inflammatory cells, fibroblasts and sensory nerve endings.

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