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$P2X_1$ and $P2X_3$ Purinergic Receptors Differentially Modulate the Inflammatory Response in Human Osteoarthritic Synovial Fibroblasts

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Key Words

P2X purinergic receptors • Osteoarthritis • Human synovial fibroblasts • TNF-α production • PGE_2 and IL-6 release

Abstract

Background/Aims: P2X receptors are membrane ion channels activated by extracellular adenosine 5'-triphosphate (ATP) which contribute to various physiological processes. The present study describes in synovial fibroblasts (SFs) obtained from osteoarthritis (OA) patients and in SW 982 cells derived from human synovial sarcoma a pharmacological characterization of $P2X_1$ and $P2X_3$ receptors implicated in the modulation of inflammatory processes in joint diseases. Methods: mRNA, western blotting, saturation and competition binding experiments were used to characterize purinergic receptors. From a functional point of view nuclear factor κB (NF-κB) activation, tumour necrosis factor-α (TNF-α), interleukin 6 (IL-6) and prostaglandin E_2 (PGE_2) production were evaluated by means of enzyme-linked immunosorbent assays. Results: $P2X_1$ and $P2X_3$ receptors were present with high affinity and density. Selected purinergic agonists and antagonists exhibited a different thermodynamic behavior. $P2X_1$ receptors showed an anti-inflammatory effect reducing NF-κB activation and TNF-α release whilst $P2X_3$ receptors mediated opposite response. No effect was mediated by $P2X_1$ and $P2X_3$ receptors on IL-6 and PGE_2 production. Conclusion: SFs from OA patients and SW 982 cells similarly express $P2X_1$ and $P2X_3$ receptors which are able to modulate in opposite way some functional responses closely associated with inflammation suggesting that purinergic receptors may represent a potential target in therapeutic anti-inflammatory joint interventions.

Introduction

Human synovial fibroblasts (SFs) play a central role in the pathogenesis of joint destruction primarily by the secretion of a wide range of pro-inflammatory mediators including cytokines, growth factors and lipid mediators of inflammation. Pro-inflammatory agents produced by SFs

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are detrimental to articular cartilage in different joint diseases such as osteoarthritis (OA) and rheumatoid arthritis [1, 2].

Purine receptors have historically been classified in two categories named as P1 and P2 purinergic receptors which were subdivided into G protein coupled P2Y receptors and P2X ligand-gated ion channels [3, 4]. Different functional responses due to these purinergic receptors have been described in a wide range of tissues and biological systems suggesting that ATP may contribute to various physiological processes [5, 6]. The effect of extracellular nucleotides, through P2 receptors, was previously reported in the regulation of bone metabolism, in the breakdown of proteoglycans and in the production of cartilage inflammatory mediators [7, 8]. The pharmacological characterization of P2 receptors and the effects of nucleotides on SFs have not been investigated in detail although it has been shown that these cells respond to extracellular ATP [7]. Synergistic interaction between IL-6, PGE_2 and the presence of purinergic receptors in SFs may be important in the modulation of the joint tissue destruction including the damage related to inflammatory pathologies [9, 10]. It is well reported that ATP was able to mediate an increase of IL-6 and TNF-α in different cell lines [11, 12]. The study of the pharmacologic modulation of the NF-κB pathways linked to p50 and p65 polypeptides suggest its involvement in the pathogenesis of several inflammatory diseases [13, 14]. The characterization of endogenous and recombinant P2X purinergic receptors by using radioligand binding techniques have had limited success. Purinergic receptor pharmacological studies have been also hampered by the lack of highly potent and selective agonists and/or antagonists [3]. The availability of [^{3}H]αβmethyleneATP ([^{3}H]αβmeATP) as radioligand to study P2X purinoceptors represents a useful tool for determining the distribution of these receptors and their binding parameters [15]. Recently, [^{3}H]αβmeATP binding and thermodynamic characterization of human $P2X_1$ and $P2X_3$ purinergic receptors in HEK 293 cells and in bovine chondrocytes revealed the possibility to obtain informations by agonist-antagonist discrimination [16, 17].

The aim of this study was to investigate the presence of P2X receptors in primary cultures of SFs from patients with OA and in SW 982 cells derived from human synovial sarcoma by using mRNA and western blotting assays. In the same substrates, saturation binding experiments were performed to evaluate affinity (K_D) and density (Bmax) of P2X receptors. Affinity values expressed as Ki or K_H and K_L of selected purinergic agonists and antagonists were determined by using competition binding experiments. A thermodynamic analysis was performed to investigate new insights into the forces driving drug-purinergic receptor coupling. In order to complete the pharmacological characterization, P2X receptors were studied from a functional point of view. The effect of selected $P2X_1$ and $P2X_3$ agonists was investigated on NF-κB and NF-IL-6 activation and on TNF-α, IL-6 and PGE_2 release. The capability of well-known purinergic antagonists to block the effect of the purinergic agonists was also carried out to better verify the involvement of the $P2X_1$ and $P2X_3$ purinergic receptors.

Materials and Methods

Materials

[^{3}H]αβmeATP, ([^{3}H]α,βmethyleneATP; specific activity 15.0 Ci/mmol) was obtained from NEN-Perkin Elmer Life and Analytical Sciences (USA). αβmeATP (αβmethyleneATP), βγmeATP (βγmethyleneATP), BzATP (benzoylATP), A317491 (5-({[3-phenoxybenzyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino}carbonyl)-1,2,4-benzenetri-carboxylic acid), vimentin, CD14 and von-Willebrand antibodies were purchased from Sigma-Aldrich Advanced Sciences (St. Louis, MO, USA). NF023 (8,8'-[carbonylbis (imino-3,1-phenylene carbonylimino)]bis(1,3,5-naphthalene-trisulfonic acid) and KN-62 (4-[(2S)-2-[(5-isoquinolinylsulfonyl) methylamino]-3-oxo- 3-(4-phenyl-1-piperazinyl) propyl] phenyl iso-quinolinesulfonic acid ester) were obtained from Tocris Cookson Ltd (Bristol, UK). NF-κB kit was purchased from Active Motif, Carlsbad, USA. TNF-α, IL-6 and PGE_2 ELISA kits were purchased from R&D Systems, Inc. Minneapolis, USA. HEK-293 cells transfected with the human recombinant $P2X_1$ and $P2X_3$ receptors were kindly provided by Prof. Annmarie Surprenant (Institute of Molecular Physiology, University of Sheffield, Sheffield, England, UK). All other reagents were of analytical grade and obtained from commercial sources.

Cell culture and membrane preparation

HEK293-h$P2X_1$ and HEK293-h$P2X_3$ cells were generated by stable transfection of human embryonic kidney cell cultures with $P2X_1$ and $P2X_3$ purinergic receptors [18]. SFs were obtained by enzymatic digestion of synovial tissues derived from 27 patients with end-stage OA undergoing total joint replacement surgery [19, 20]. The diagnosis was based on clinical and radiological criteria. Human samples were collected with approved informed consent in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the local Ethics Committee of the University of Ferrara and the subjects provided written consent after receiving detailed verbal and written explanations of the study. SW 982 cells derived from a human synovial sarcoma are SFs obtained from American Type Culture Collection (Bethesda, MD) [21].

To avoid the degradation of nucleotides, cells were

incubated in serum-free medium during the pharmacological treatment with purinergic agonists and/or antagonists investigated.

SFs characterization

Immunofluorescence with the primary monoclonal antibody specific for the human vimentin was used to evaluate the expression of vimentin, a fibroblast marker, in SW 982 and in primary cultures of human SFs, as previously described [16, 19]. Human SFs and SW 982 were maintained in culture in DMEM, 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM). Human SFs when reaching confluence were passaged and used at the 3rd-4th passages for binding and functional experiments. Nuclei were stained with the selective DNA dye, DAPI (4',6-Diamidino-2-phenylindole) (0.1 mg/ml in PBS-EGTA) for 10 minutes. Fluorescence was visualized using the Nikon Eclipse TE 2000-E microscope equipped with a digital camera (DXM 1200F, Nikon Instruments Spa, Sesto Fiorentino, Firenze, Italy). Monoclonal antibodies to CD14 and von-Willebrand factor were also used to exclude the presence of contaminating macrophages or endothelial cells.

Real-Time RT-PCR experiments

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method [22]. Quantitative real-time RT-PCR assay of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$ mRNAs was carried out using gene-specific fluorescently labelled TaqMan MGB probe (minor groove binder) in a ABI Prism 7700 Sequence Detection System (Applied Biosystems). Human GAPDH was used as a reference gene.

Western blotting analysis

Primary cultures of human SFs and SW 982 cells in comparison with HEK293-h$P2X_1$ and HEK293-h$P2X_3$ cells were harvested and washed with ice-cold PBS containing 1 mM sodium orthovanadate, 104 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, 1.4 mM E-64 [23]. Aliquots of total protein sample (50 µg) were analyzed using antibodies specific for human $P2X_1$ and $P2X_3$ purinergic receptors (1 µg/ml dilution). Filters were washed and incubated for 1 hour at room temperature with peroxidase-conjugated secondary antibodies (1:2000 dilution). Specific reactions were revealed with Enhanced Chemiluminescence Western blotting detection reagent (Amersham Biosciences). β-actin was used as a loading control. Additional western blotting analysis was also performed for the NF-IL-6 protein expression as previously described [24].

Saturation and competition binding assays

Saturation and competition binding assays were carried out in primary cultures of SFs and in SW 982 membranes at 5, 10, 15, 20, 25 and 30°C. Saturation binding experiments of [^{3}H]αβmeATP (from 0.1 to 50 nM) were performed by using the membranes previously obtained (100 µg protein/assay). Previous time course experiments showed that [^{3}H]αβmeATP binding reached equilibrium after approximately 15 min and was stable for at least 3 hours. The incubation times used in binding experiments were: 40 min at 5°C, 50 min at 10°C, 60 min at 15°C, 70 min at 20°C, 80 min at 25°C and 90 min at 30°C.

Competition experiments of 3 nM [^{3}H]αβmeATP were conducted incubating membranes (100 µg protein/assay) and 13 different concentrations of purinergic agonists or antagonists examined for thermodynamic studies. Non specific binding was determined in the presence of 1 µM αβmeATP [16, 17]. Bound and free radioactivity were separated by rapid filtration through Whatman GF/B glass-fiber filters using a Brandel instrument and the radioactivity was counted on a Scintillation Counter Tri Carb Packard 2500 TR.

Thermodynamic data determination

For a generic binding equilibrium L+R = LR (L = ligand, R = receptor) the affinity association constant $K_A = 1/K_D$ is directly related to the standard free energy $\Delta G°$ ($\Delta G° = -RT\ln K_A$) which can be separated in its enthalpic and entropic contributions according to the Gibbs equation: $\Delta G° = \Delta H° - T\Delta S°$ [25].

NF-κB activation in human SFs

The NF-κB activation in nuclear extracts from human SFs and SW 982 cells was evaluated by detecting phosphorylated p65 and p50 proteins in nuclear extracts using the TransAM NF-κB kit (Active Motif, Carlsbad, USA) [26]. Phosphorylated NF-κB subunits specifically bind to the immobilized oligonucleotides containing the NF-kB consensus site (5'-GGGACTTTCC-3'). The primary antibody used to detect NF-κB recognized an epitope on each subunit that is accessible only when activated and bound to its DNA target. A horseradish peroxidase (HRP)-conjugated secondary antibody provided a sensitive colorimetric readout that was quantified by spectrophotometry at 450 nm.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of TNF-α, IL-6 and PGE_2 was measured using commercially available competitive enzyme immunoassays (R&D Systems, Minneapolis, USA) in duplicate samples or standards [27, 28].

Data and statistical analysis

The protein concentration was determined according to a Bio-Rad method with bovine albumin as reference standard [29]. A weighted non linear least-squares curve fitting program Ligand was used for computer analysis of saturation and competition binding experiments [30]. Analysis of data, expressed as mean±SEM, was performed by one-way analysis of variance. Differences were analyzed with Dunnett's test and were considered significant at a value of $p<0.01$.

Results

Phenotype characterization of SFs

SFs isolated from synovial of OA patients showed to be a homogenous population as demonstrated by their fibroblast-like morphology. Primary cultures of SFs (Fig. 1a) and SW 982 cells (Fig. 1b) also showed the expression

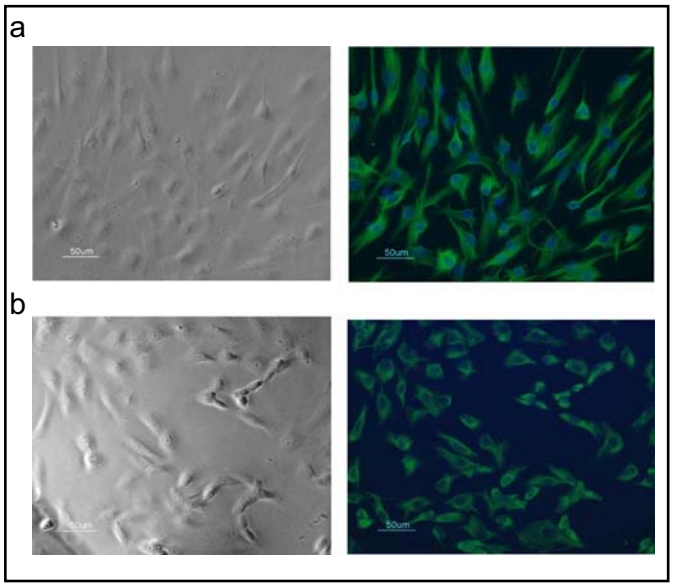

Fig. 1. Culture of human SFs (a) and SW 982 cells (b). Left panels: phase contrast. Right panels: vimentin expression by immunofluorescence. Nuclei were counterstained in blue with DAPI.

of vimentin, a specific cellular marker for mesenchymal cells and SFs. No stain was observed with MoAbs, to CD14 and von-Willebrand factor indicating the absence of contaminating cells (data not shown).

Evaluation of P2X purinergic receptor mRNA expression and protein level

The expression of $P2X_{1-7}$ mRNA was shown and high levels of $P2X_1$, $P2X_3$ and $P2X_7$ mRNA were found in human SFs and SW 982 cells (Fig. 2a). The presence of $P2X_1$ and $P2X_3$ was confirmed by western blotting in comparison with HEK293-h$P2X_1$ and HEK293-h$P2X_3$ cells (Fig. 2b). Densitometric analysis of the bands obtained was also performed as shown in Fig. 2c,d.

Saturation and competition binding experiments

Saturation binding experiments in primary cultures of human SFs (Fig. 3a,b) and in SW 982 (Fig. 3c,d) membranes were carried out to evaluate affinity (K_D) and receptor density (Bmax) values of P2X receptors. Binding parameters were determined at various temperatures (5, 10, 15, 20, 25 and 30°C) by using [^{3}H]αβmeATP as radioligand and showed that K_D values changed with temperature (from 3 to 9 nM, respectively) and Bmax values in the picomolar range appeared to be largerly independent of it. Saturation binding experiments, performed at 5°C, in human SFs demonstrated the presence of an high affinity binding site (K_D=3.1±0.3 nM)

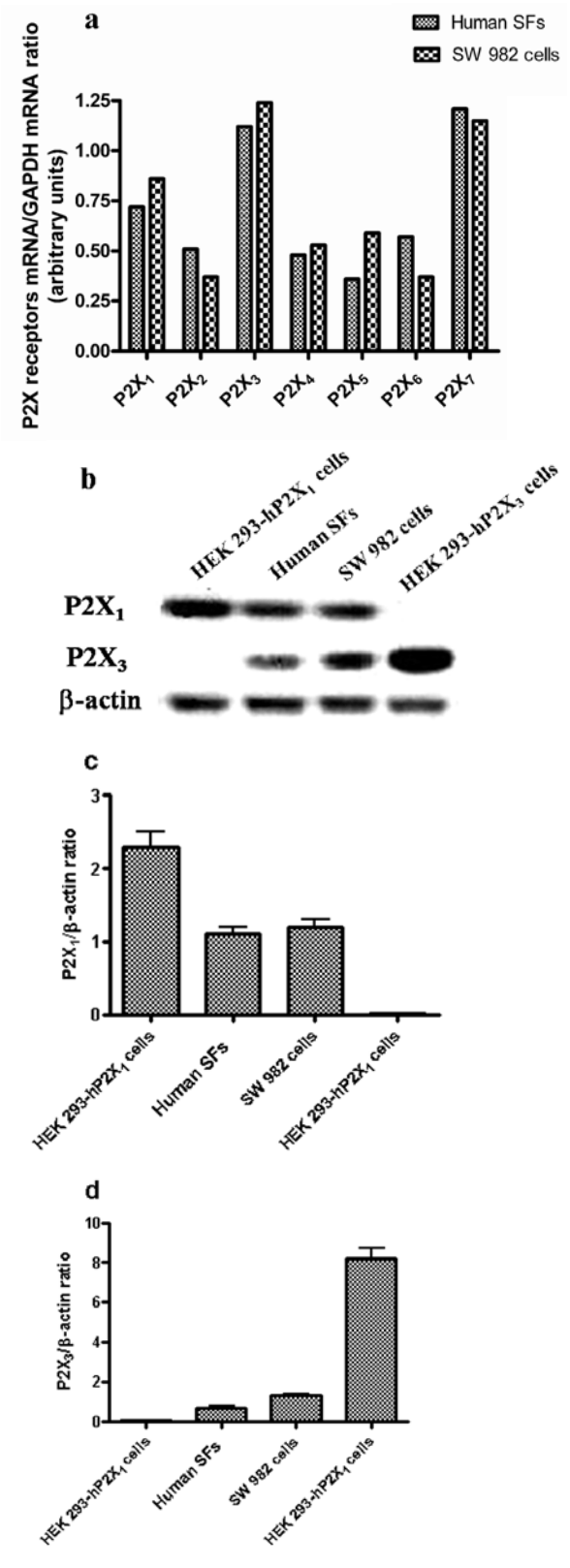

Fig. 2. mRNA expression of P2X purinergic receptors (a) and western blotting analysis (b) in human SFs and SW 982 cells in comparison with HEK293-h$P2X_1$ and HEK293-h$P2X_3$ cells (n=3 independent experiments). Densitometric analysis for h$P2X_1$ and h$P2X_3$ purinergic receptors are shown (c,d).

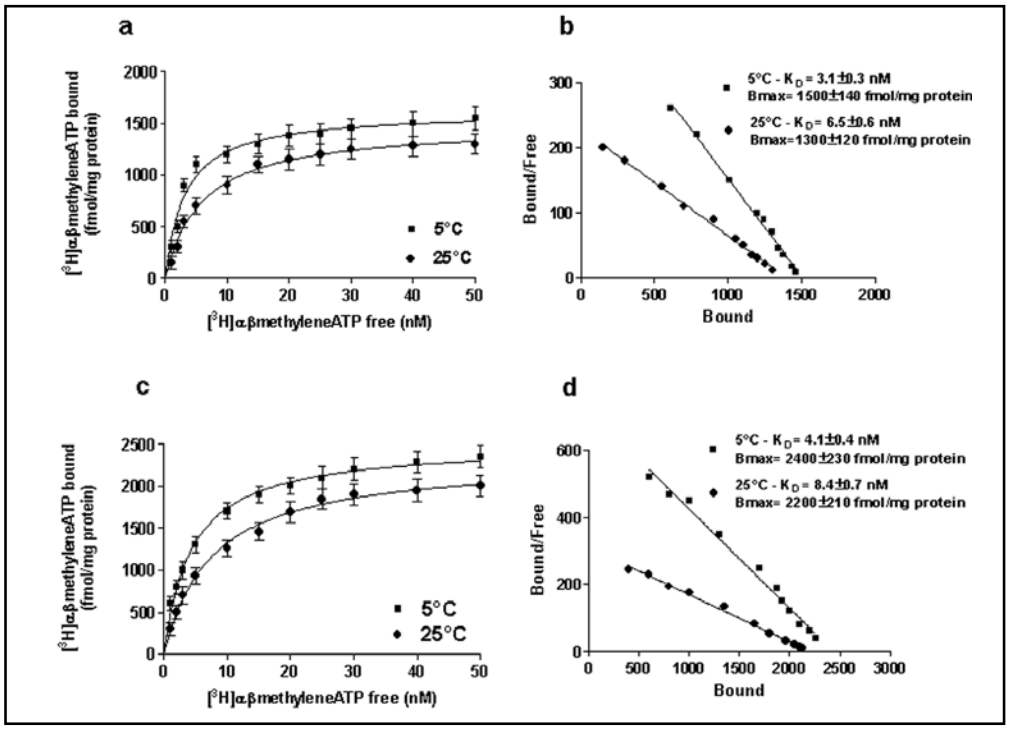

Fig. 3. Saturation curves and Scatchard plot of [^{3}H]αβmeATP binding to P2X purinergic receptors in human SFs (a,b) and SW 982 (c,d) membranes at 5°C and 25°C. Each value represents the mean ± SEM of 4 separate experiments.

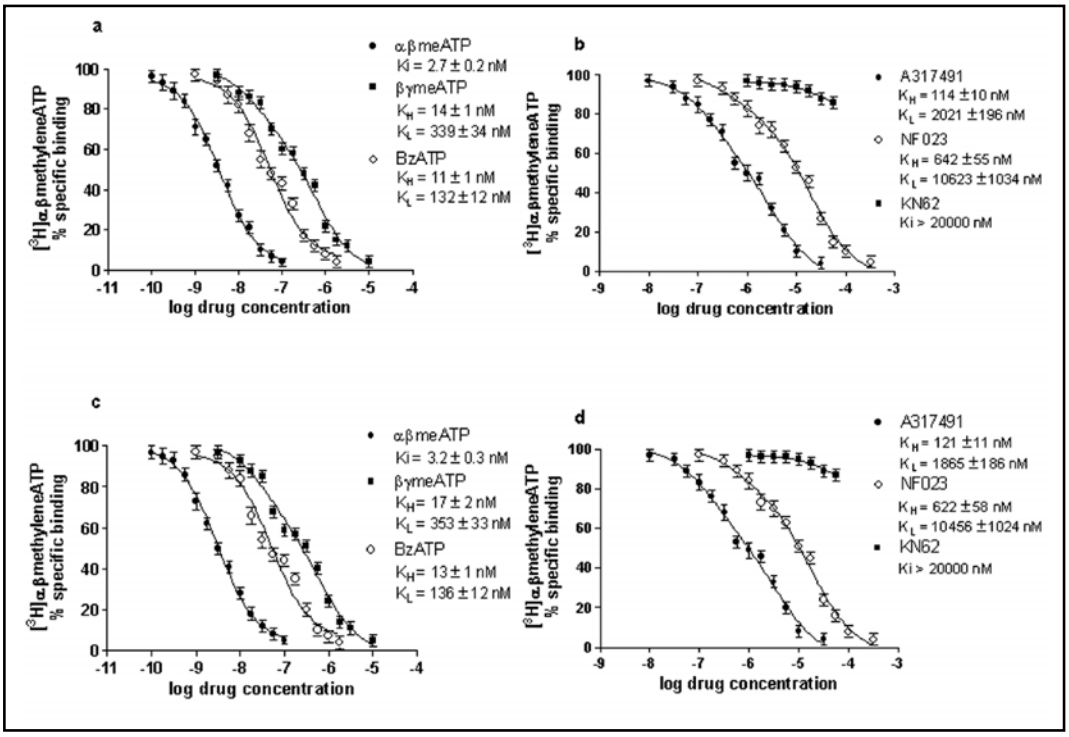

Fig. 4. Competition curves of selected purinergic agonists and antagonists in human SFs (a,b) and in SW 982 (c,d) membranes. Each value represents the mean ± SEM of 4 separate experiments.

and a receptor density (Bmax) of 1500±140 fmol/mg protein. Similarly, SW 982 cells showed K_D value of 4.1±0.4 nM and Bmax values of 2400±230 fmol/mg protein. With the increase of the temperature, affinity values decreased (K_D=6.5±0.6 nM, K_D=8.4±0.7 nM, at 25°C, respectively) (Fig. 3b,d).

Affinity values obtained in [^{3}H]αβmeATP competition binding experiments by using selected purinergic agonists and antagonists in human SFs membranes are shown in Table 1. In human SFs membranes affinity values of the purinergic ligands used in functional experiments were obtained incubating 13 different concentration of purinergic agonists (Fig 4a) or antagonists (Fig 4b). Interestingly, βγmeATP, BzATP, A317491 and NF 023 showed a better fit for a two binding site model, suggesting a different affinity for $P2X_1$ and

Table 1. Affinities, expressed as Ki or K_H and K_L values (nM) of selected purinergic agonists and antagonists in human SFs (A) and SW 982 cells (B) at different temperatures. Data are expressed as the mean ± SEM of 4 independent experiments performed in duplicate.

Ligand	5 °C 278 K	10 °C 283 K	15 °C 288 K	20 °C 293 K	25 °C 298 K	30 °C 303 K
(A)						
Agonists						
αβmeATP						
Ki (nM)	2.7±0.3	3.5±0.3	4.6±0.5	5.8±0.6	6.5±0.6	7.4±0.7
βγmeATP						
K_H (nM)	14±1	17±2	19±2	24±2	31±3	38±3
K_L (nM)	339±34	395±37	452±41	526±48	597±53	671±64
BzATP						
K_H (nM)	11±1	13±1	16±1	20±2	24±2	28±3
K_L (nM)	132±11	163±15	195±20	239±22	286±27	358±32
Antagonists						
A317491						
K_H (nM)	114±10	102±9	89±8	74±7	56±6	43±5
K_L (nM)	2021±190	2817±265	3381±323	3852±374	4268±413	4815±468
NF023						
K_H (nM)	642±63	823±77	992±91	1076±105	1184±113	1328±129
K_L (nM)	10623±1054	9223±892	8412±826	7636±697	6844±672	5729±536
KN62						
Ki (nM)	>20000	>20000	>20000	>20000	>20000	>20000
(B)						
Agonists						
αβmeATP						
Ki (nM)	3.2±0.3	3.8±0.4	5.2±0.5	6.3±0.5	7.0±0.6	7.9±0.6
βγmeATP						
K_H (nM)	17±1	19±2	20±2	25±2	32±3	40±3
K_L (nM)	353±31	403±39	464±42	538±51	611±57	693±63
BzATP						
K_H (nM)	13±1	15±1	18±2	21±2	26±2	30±3
K_L (nM)	136±12	171±16	198±18	245±21	293±28	365±35
Antagonists						
A317491						
K_H (nM)	121±11	105±10	93±9	78±8	59±6	48±5
K_L (nM)	1865±172	2724±213	3243±311	3726±353	4115±391	4737±424
NF023						
K_H (nM)	622±59	815±73	983±88	1052±97	1148±102	1296±116
K_L (nM)	10456±1023	9185±871	9356±794	7518±702	6738±623	5612±513
KN62						
Ki (nM)	>20000	>20000	>20000	>20000	>20000	>20000

$P2X_3$ purinergic receptors. On the contrary, αβmeATP showed a better fit for a one site binding model, confirming a similar affinity for $P2X_1$ and $P2X_3$ purinergic receptors. KN62 was not able to displace [^{3}H]αβmeATP (Ki > 20 μM). Similar affinity values were also obtained in SW 982 cells for purinergic agonists (Fig 4c) and antagonists (Fig 4d). In human SFs and in SW982 cells, the affinities of αβmeATP, βγmeATP and BzATP were decreased with the increase of the temperature (from 5 to 30°C) as reported in Table 1. Interestingly, the increase of the temperature differentially modulated the affinity of the antagonists and mediated: i) the reduction in K_H of A317491 and in K_L of NF023; ii) the increase in K_L of A317491 and in K_H of NF023 (Table 1).

Thermodynamic analysis

The van't Hoff plots for purinergic ligands examined were essentially linear in the range 5-30°C in human SFs (Fig. 5a,b) and in SW 982 cells (Fig. 5c,d).

Thermodynamic parameters (ΔG°, ΔH°, ΔS°) were reported in Table 2. The standard free energy was calculated as $\Delta G° = -RT\ln K_A$ at 298.15 K, the standard enthalpy, ΔH°, from the van't Hoff plot $\ln K_A$ versus (1/T) (the slope of which is -ΔH°/R) and the standard entropy as $\Delta S° = (\Delta H° - \Delta G°)/T$ with T = 298.15 K and R = 8.314 J/K/mol). K_A values were obtained from saturation experiments of [^{3}H]αβmeATP binding to SF membranes carried out at 0, 10, 15, 20, 25 and 30°C in a thermostatic bath assuring a temperature of ± 0.1°C. The

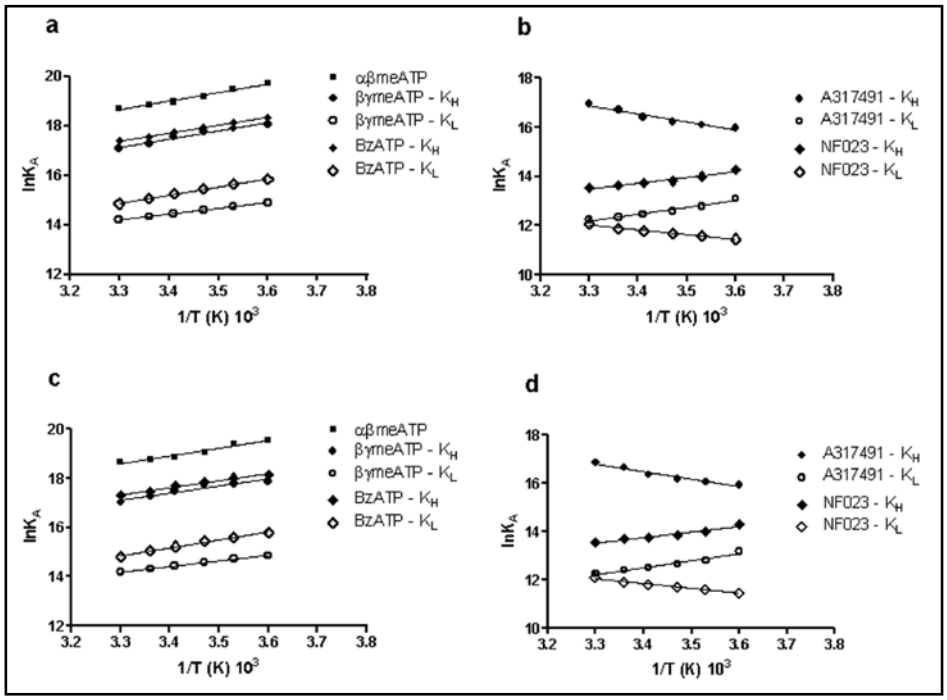

Fig. 5. Van't Hoff plots in human SFs (a,b) and in SW 982 (c,d) membranes showing the effect of temperature on the equilibrium binding association constant, K_A, for selected P2X purinergic agonists (a,c) and antagonists (b,d). The plots are essentially linear in the temperature range investigated (5-30°C).

Table 2. Thermodynamic parameters for the binding equilibrium of [^{3}H]αβmeATP in human SFs (A) and SW982 cells (B) of selected purinergic agonists and antagonists. Themodynamic parameters: ΔG°, ΔH° and ΔS° values are given at 278 K. NC= not calculated.

Ligand	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol/K)
(A)			
Agonists			
αβmeATP			
Ki	-46.7±0.2	-28.8±2.3	60.0±5.8
βγmeATP			
K_H	-42.9±0.2	-27.9±2.2	50.4±5.3
K_L	-35.5±0.1	-19.4±1.9	54.1±4.9
BzATP			
K_H	-43.5±0.2	-26.9±2.1	55.6±5.7
K_L	-37.3±0.1	-27.6±2.4	32.6±3.6
Antagonists			
A317491			
K_H	-41.4±0.2	27.2±2.5	230.1±22.4
K_L	-30.6±0.1	-23.2±2.1	24.7±2.2
NF023			
K_H	-33.8±0.1	-19.5±1.9	48.1±4.7
K_L	-29.5±0.1	16.4±1.7	153.9±13.5
KN62			
Ki	> -26.8	NC	NC
(B)			
Agonists			
αβmeATP			
Ki	-46.5±0.2	-26.3±2.4	67.8±6.1
βγmeATP			
K_H	-42.8±0.2	-24.2±2.1	62.4±5.9
K_L	-35.4±0.1	-19.1±2.0	54.9±5.2
BzATP			
K_H	-43.3±0.2	-24.0±2.2	64.7±6.3
K_L	-37.2±0.1	-27.2±2.3	33.8±3.4
Antagonists			
A317491			
K_H	-41.3±0.2	26.0±2.4	225.6±23.5
K_L	-30.7±0.1	-24.4±1.9	21.1±2.1
NF023			
K_H	-33.9±0.1	-19.2±1.8	49.3±4.6
K_L	-29.6±0.1	16.6±1.7	154.5±14.2
KN62			
Ki	> -26.8	NC	NC

slopes of van't Hoff plots were positive for the purinergic agonists examined whose affinities decreased with increase of the temperature. Different behavior was reported for the purinergic antagonists. In fact, the slopes of van't Hoff plots for the K_H of A317491 and the K_L of NF023 were negative whose affinities are improved by an increase of the temperature. In contrast, the slopes of van't Hoff plots for K_L of A317491 and K_H of NF023 were positive. Final thermodynamic parameters (expressed as mean values ± standard error of 4 independent experiments) of the selected purinergic compounds revealed that the binding of agonists was enthalpy and entropy driven (Table 2). In addition, high affinity of A317491 and low affinity of NF023 were associated with an entropy driven binding whilst low affinity of A317491 and high affinity of NF023 showed an enthalpy and entropy driven binding (Table 2).

Transcription factors in human fibroblast-like synoviocytes

NF-κB levels were evaluated studying P50 and P65 subunits activation. In human SFs, αβmeATP ($P2X_1$ and $P2X_3$ agonist) and BzATP ($P2X_7$ agonist) were able to increase by 85% and 152% the basal level of P65 subunit,

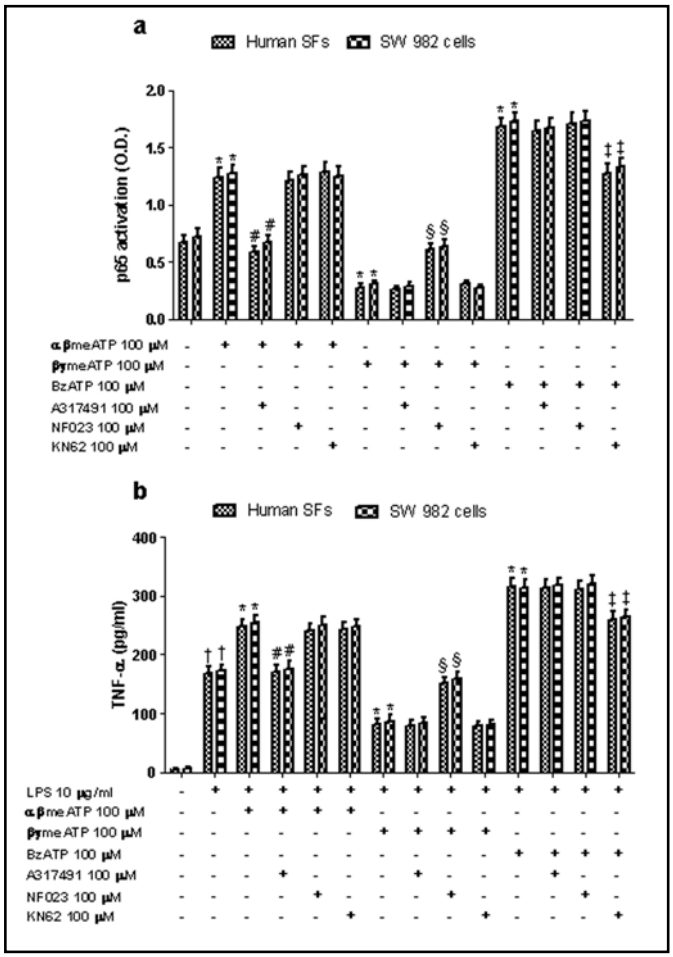

Fig. 6. Effect of αβmeATP, βγmeATP and BzATP (100 μM) in human SFs and in SW 982 cells on NF-κB activation by detecting phosphorylated p65 (a) in nuclear extracts and on TNF-α levels in control conditions and stimulated by LPS (10 μg/ml) (b). The effect of purinergic antagonists were also studied (n=4 independent experiments). (a): *, $p<0.01$ versus control conditions; #, $p<0.01$ versus αβmeATP; §, $p<0.01$ versus βγmeATP; ‡, $p<0.02$ versus BzATP. (b) †, $p<0.01$ versus control conditions; *, $p<0.01$ versus LPS; #, $p<0.01$ versus αβmeATP; §, $p<0.01$ versus βγmeATP; ‡, $p<0.02$ versus BzATP.

respectively. βγmeATP ($P2X_1$ agonist) reduced by 58% the basal level of P65 subunit (Fig. 6a). The capability of typical purinergic antagonists were investigated using A317491 and NF 023 which are able to block the effect of αβmeATP and βγmeATP to control values, respectively. A317491 or NF 023 were not able to counteract the effect of βγmeATP or αβmeATP, respectively. In addition, a $P2X_7$ antagonist, KN 62 partially reduced the effect of BzATP and was not able to counteract the effect of βγmeATP or αβmeATP. Similar results were also obtained in SW 982 cells confirming that $P2X_1$ and $P2X_3$ purinergic receptors

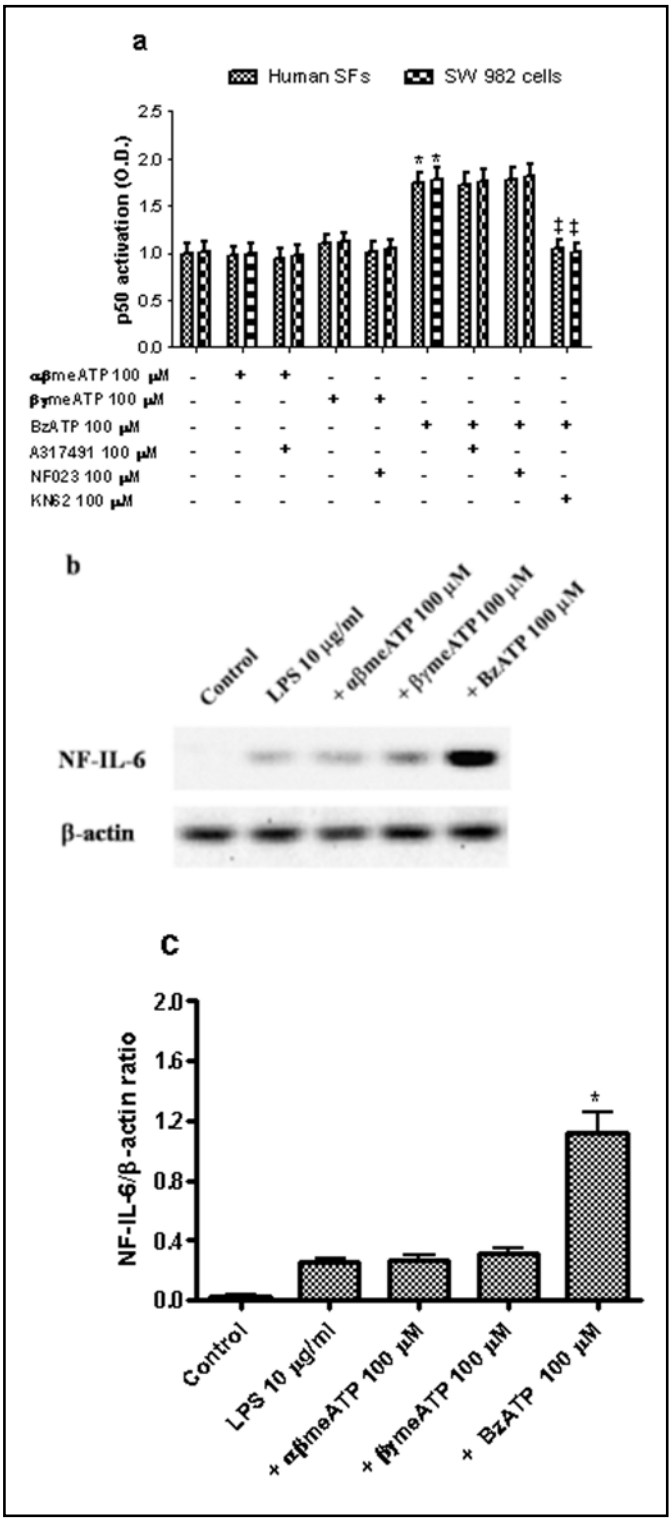

Fig. 7. Effect of αβmeATP, βγmeATP and BzATP (100 μM) in human SFs and in SW 982 cells on NFκB activation by detecting phosphorylated p50 in nuclear extracts (a): *, $p<0.01$ versus control conditions; ‡, $p<0.01$ versus BzATP. No effect was present in western blotting analysis by αβmeATP and βγmeATP (100 μM) in human SFs on NF-IL-6 activation. BzATP (100 μM) was able to statistically increase NF-IL-6 activation (b). Densitometric analysis for NF-IL-6 was shown (c): *, $p<0.01$ versus control conditions (n=4 independent experiments).

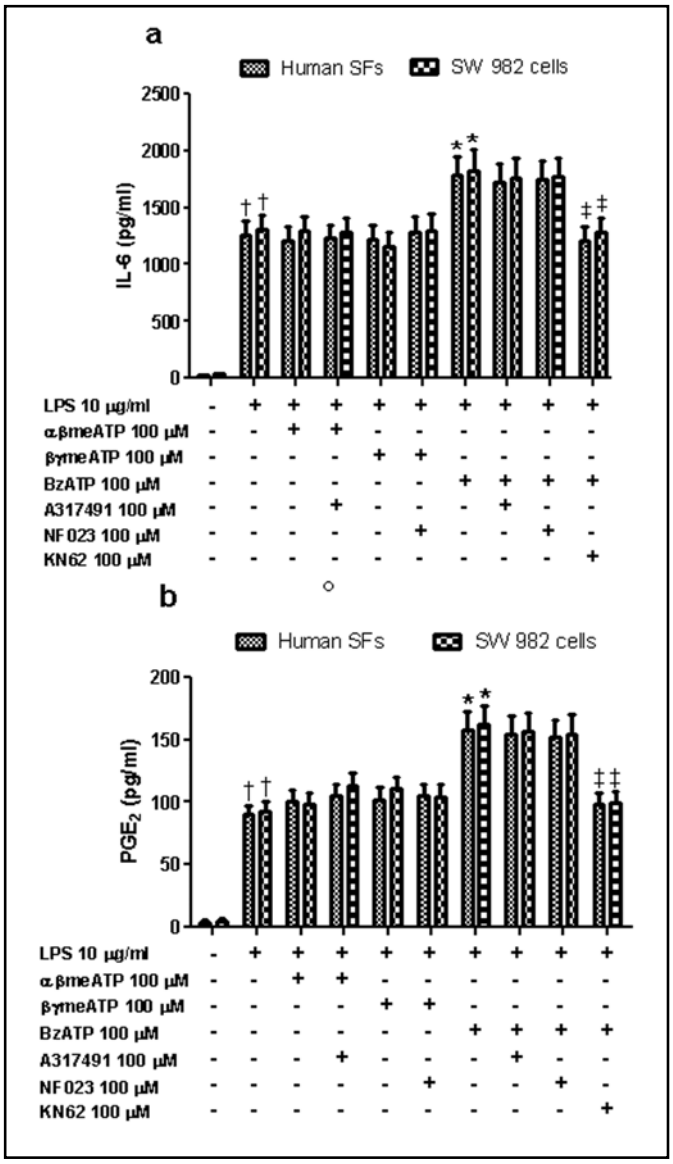

Fig. 8. Effect of αβmeATP, βγmeATP and BzATP (100 μM) in human SFs and in SW 982 cells on IL-6 (a) and PGE_2 (b) production in control conditions and stimulated by LPS (10 μg/ml). The effect of purinergic antagonists were also evaluated (n=4 independent experiments). †, $p<0.01$ versus control conditions; *, $p<0.01$ versus LPS; ‡, $p<0.01$ versus BzATP.

present in human SFs were closely similar to those observed in this cell line.

No modulation by purinergic agonists and antagonists was found on P50 subunit activation and NF-IL6 transcription factor with the exception of BzATP that was able to increase the basal level of P50 subunit and NF-IL-6 transcription factor. This effect was blocked only by KN-62 and not by A317491 or NF 023, suggesting the involvement of $P2X_7$ receptors (Fig. 7).

TNF-α, IL-6 and PGE_2 production

In both OA SFs and SW cells the effect of selected purinergic agonists and antagonists was evaluated in the presence of LPS (10 μg/ml) specific stimulation in TNF-α release (Fig. 6b), in IL-6 (Fig. 8a) and PGE_2 production (Fig. 8b). The stimulation of $P2X_1$ and $P2X_3$ receptors mediated a decrease and an increase of TNF-α release, respectively. No effect was present on the modulation of IL-6 and PGE_2 production by the stimulation of these purinergic receptors (Fig. 8a,b). In human SFs, αβmeATP was able to significantly increase TNF-α production by 47%. $P2X_1$ stimulation by using βγmeATP revealed a significant decrease by 51%. In the same experimental conditions, the effect of BzATP was to increase by 88% the TNF-α release. A317491 (100 μM) counteracted the effect of αβmeATP but not of βγmeATP. NF023 (100 μM) blocked the effect of βγmeATP but not of αβmeATP. In addition, evaluating the effect of the three purinergic antagonists, only KN 62 was able to decrease partially the BzATP-mediated increase of TNF-α production.

Discussion

Nowadays no specific therapy, based on intracellular pathways of chondrocytes and/or SFs, exists for the medical management of OA. Although different cytokines provide a potential therapy for OA it will be necessary to elucidate novel targets to be employed in this disease [31]. The purpose of the present paper was to document the presence, the binding parameters and the functionality of $P2X_1$ and $P2X_3$ receptors in SFs derived from OA patients and in SW 982 cells used as a representative model of human synovial cell line. These data could be very important considering that synovial tissue is known to have a key role as target tissue in the joint during inflammation and in the pathophysiology of OA [32, 33]. In this paper we report for the first time a binding and functional characterization of P2X purinergic receptors in human synoviocytes. No works are present in the literature showing the presence and the role of $P2X_1$ and $P2X_3$ purinergic receptors in human synoviocytes.

In human SFs the presence of purinergic receptors was investigated through mRNA and western blotting analysis. To quantify exactly the affinity and density of the P2X receptors investigated, saturation binding studies were performed revealing that the affinity (K_D, nM) was in the nanomolar range and the receptor density (Bmax, fmol/mg protein) was very high. The binding was rapid, reversible, saturable and indicated similar parameters in

both human SFs and in SW 982 cells. In addition, as demonstrated by a previous work from our group, αβmeATP was able to label with a similar affinity both $P2X_1$ and $P2X_3$ purinergic receptors expressed in HEK 293 cells [17]. These results were also confirmed by competition binding experiments where αβmeATP showed a closely similar affinity for human $P2X_1$ and $P2X_3$ purinergic receptors expressed in HEK 293 cells. On the basis of these previous results, we can hypothesis that in saturation binding experiments performed in human synoviocytes, [^{3}H]-αβmeATP labeled both $P2X_1$ and $P2X_3$ purinergic receptors with similar affinity. Competition binding experiments revealed that αβmeATP have higher affinity, in the nanomolar range, than other agonists and antagonists. To evaluate if the ligands used in functional experiments had different affinity values for $P2X_1$ and $P2X_3$ receptors, competition binding experiments with 13 various concentrations of these compounds were performed. As expected, αβmeATP revealed a monophasic curve showing the best fit for a receptor population constituted from $P2X_1$ and $P2X_3$ with similar affinity. Interestingly, βγmeATP, BzATP, A317491 and NF 023 showed a best fit for a two site binding model, suggesting that these compounds have a different affinity for $P2X_1$ and $P2X_3$ purinergic receptors. From these results we can hypothesis that K_H for βγmeATP and NF 023 represent their affinity versus $P2X_1$ purinergic receptors whilst BzATP and A317491 were able to bind with high affinity $P2X_3$ purinergic subtypes.

We have also performed competition experiments at six different temperatures that could represent an useful *in vitro* experimental approach to discriminate agonists from antagonists by means of binding experiments. Thermodynamic parameters obtained from the van't Hoff plots in the cells examined indicated that αβmeATP binding to purinergic receptors is enthalpy- and entropy-driven, with a major contribution of the enthalpic component. The presence of the linearity of van't Hoff plots for P2X receptors similarly to those verified for other membrane receptors showed that ΔH° and ΔS° values are independent of temperature [16, 17]. These results provide further informations to the several data present in literature showing the thermodynamic parameters for a remarkable number of ligands and receptors that allow a precise investigation at molecular level on the role played during the binding by ligand substituents and by receptor aminoacids [25, 34, 35]. We previously reported that $P2X_3$ purinergic receptors are thermodynamically discriminated whilst $P2X_1$ receptor subtypes are not discriminated [16]. For human $P2X_1$ receptors expressed in HEK293 cells, purinergic agonists and antagonists showed an enthalpy-entropy driven binding. In HEK293-h$P2X_3$ cells only the agonists had an enthalpy-entropy driven binding whilst the antagonists showed an entropy driven binding. The novel results obtained in human SFs and SW 982 cells, suggest that the purinergic agonists examined have an enthalpy-entropy driven binding. In addition, the purinergic antagonists such as A137491 and NF023 showed a different thermodynamic behavior. In fact, high affinity binding site for A137491 was closely associated to the typical $P2X_3$ thermodynamic behavior as previously reported in HEK293 cells. The high affinity binding site of NF023 was similar to the thermodynamic behavior of $P2X_1$ receptors [16]. These experimental data in human SFs and in SW 982 cells confirm that $P2X_3$ receptors are thermodynamically discriminated and $P2X_1$ receptors are not discriminated.

Another purpose of the present study was to investigate the purinergic receptor functional activities in human SFs. To this aim, the effect on NF-κB activation and on the release of pro-inflammatory factors such as TNF-α suggested the possible involvement in inflammatory process of $P2X_1$ and $P2X_3$ agonists and/or antagonists. These experiments demonstrated an anti-inflammatory effect of $P2X_1$ receptors and a pro-inflammatory effect of $P2X_3$ receptors as confirmed by the contrasting effect of αβmeATP and βγmeATP in NF-κB activation and TNF-α release. These data are in agreement with those reported in literature regarding NF-κB that is one of the most important signalling pathways able to regulate pro-inflammatory cytokines such as TNF-α [36]. Interestingly, IL-6 and PGE_2 are not modulable by the presence of $P2X_1$, $P2X_3$ but only by the presence of $P2X_7$ receptors confirming previous data obtained in rheumatoid synoviocytes where is evident the involvement of $P2X_7$ purinergic receptors [10]. These results on IL-6 and PGE_2 production demonstrated a direct link in human SFs of different nuclear transcription factors involved in the modulation of pro-inflammatory molecules. In fact, NF-κB-p50 and NF-IL-6 proteins, that binds responsive elements in the IL-6 and PGE_2 gene promoters, were not modified by $P2X_1$ and $P2X_3$ agonists [14, 37]. Finally, binding, thermodynamic and functional data demonstrated that $P2X_1$ and $P2X_3$ receptors present in human SFs and in SW 982 cells are closely similar and suggested the potential use of this cell line as an *in vitro* model to study purinergic receptors from a pharmacological point of view.

In conclusion, the novel findings carried out in human SFs from OA patients and in SW 982 cells are represented

by the presence of high density of the $P2X_1$ and $P2X_3$ purinergic receptors. The functional results revealed the direct and contrasting involvement of $P2X_1$ and $P2X_3$ purinergic receptors which could represent potential mediators in the complex pathways regulating inflammatory processes in SFs suggesting their potential beneficial target for the treatment of inflammatory joint diseases.

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