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PHARMACOLOGICAL CHARACTERIZATION OF PURINE RECEPTORS IN BOVINE AND HUMAN CHONDROCYTES AND FIBROBLAST-LIKE SYNOVIOCYTES

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TABLE OF CONTENTS

GENERAL INTRODUCTION

Extracellular purines (adenosine, ATP and ADP) and pyrimidines (UTP, UDP) are important molecules which mediate several biological effects through the interaction with specific membrane receptors named purinergic receptors. Purinergic receptors are divided in P1 and P2 receptors (Figure 1); P1 receptors preferentially are able to bind adenosine while P2 receptors interact with ATP, ADP, UTP and UDP. P1 adenosine receptors are divided in four subtypes named A_1, A_{2A}, A_{2B} and A_3 that are coupled to G-protein. P2 receptors are divided into ionotropic P2X and metabotropic P2Y. Purines and pyrimidines through these receptors were involved in different processes like neurotransmission, exocrine and paracrine secretion, immunitary response, inflammation and pain.

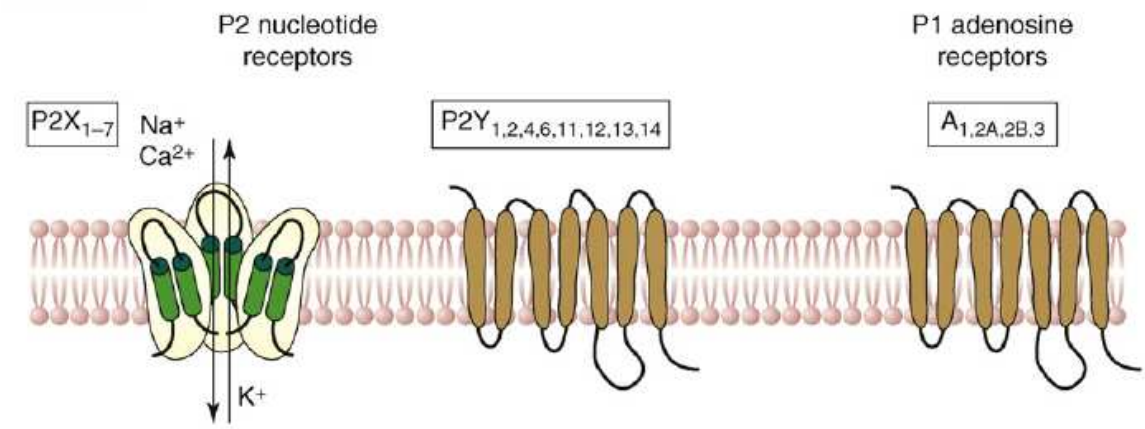

Figure 1: structure of purinergic receptors

ADENOSINE

Adenosine is a nucleoside composed of adenine attached to a ribose sugar molecule (ribofuranose) via a β-N9-glycosidic bond (Figure 2).

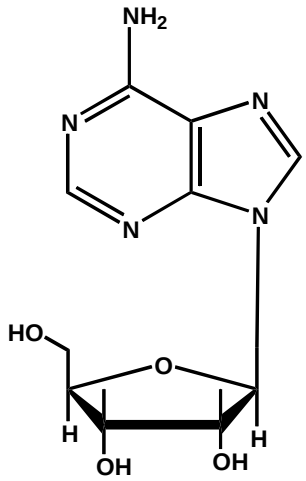

Figure 2: chemical structure of adenosine

Adenosine is an endogenous nucleoside-signalling molecule, which by acting on specific membrane receptors produces a number of physiological and pathophysiological effects in both central nervous system and peripheral organs. Adenosine is not a classical neurotransmitter because it is not principally produced and released vesicular in response to neuronal firing. Most tissues in the body and cells release adenosine to the extracellular medium, acting as an autacoid on the adenosine receptors (ARs). The basal levels of extracellular adenosine have been estimated as roughly 100 nM in the heart and 20 nM in the brain, which would only partially activate the ARs present (Fredholm et al., 2005). In the case of severe ischemic stress, the levels can rapidly rise to the micromolar range, which would cause a more intense and generalized activation of the ARs. Extracellular adenosine may arise from intracellular break-down of the adenine nucleotides, such as ATP, outside the cell. Adenosine, which is present in higher concentration inside than outside the cell, does not freely diffuse across the cell membrane. There are nucleoside transporters, such as the

equilibrative nucleoside transporter 1 (ENT1) which bring it to the extracellular space. Extracellular nucleotides may also originate from cytosolic sources, including by vescicular release exocytosis, passage through channels and cell lysis. Ectonucleotidases break down the adenine nucleotides in stages to produce free extracellular adenosine at the terminal step (Zimmermann, 2000). For example, the extracellular enzyme ectonucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1) converts ATP and ADP to AMP. The final critical step, with respect to AR activation, of conversion of AMP to adenosine is carried out by ecto-5'-nucleotidase, also known as CD37. The adenosine produced extracellulary is also subject to metabolic breakdown by adenosine deaminase to produce inosine or (re)phosphorylation by adenosine kinase to produce AMP. Therefore, when an organ is under stress there is a highly complex and time-dependent interplay of the activation of many receptors in the same vicinity. In addition to the direct activation of ARs by selective agonists or their blockade by selective antagonists, inhibition of the metabolic or transport pathways surrounding adenosine is also being explored for therapeutic purposes (McGaraughty et al., 2005).

P1 Purinergic Receptors

Adenosine act on cell surface receptors that are coupled to intracellular signaling cascades. There are four subtypes of G-protein-coupled receptors (GPCRs) named A_1, A_{2A}, A_{2B} and A_3 ARs. The second messengers associated with the ARs are historically defined with respect to the adenylate cyclase system (Fredholm and Jacobson, 2009). The A_1 and A_3 ARs inhibit the production of cyclic AMP (cAMP) through coupling to Gi. The A_{2A} and A_{2B} subtypes are coupled to Gs or Go to stimulated adenylate cyclase. Furthermore, the A_{2B} subtypes are coupled to Gq (Ryzhov et al., 2006) and it has the lowest affinity (Ki > 1 μM). Adenosine has the highest affinity at the A_1 and A_{2A} ARs (Ki values in binding of 10-30 nM at the high affinity sites) and the affinity of adenosine at the A_3 AR is intermediate (ca. 1 μM) (Jacobson et al., 1995a). The ARs, as GPCRs, share the structural motif of a single polypeptide chain forming seven transmembrane helices (TMs), with the N-terminous being extracellular and the C-terminous being cytosolic (Costanzi et al., 2007). These helices, consisting of 25-30 amino acid residues

each, are connected by six loop, three intracellular (IL) and three extracellular (EL). The extracellular regions contains sites for posttranslational modifications, such as glycosylation. The A_1 and A_3 ARs also contains sites palmitoylation in the C-terminal. The A_{2A} AR has a long C-terminal segment of more than 120 amino acid residues, which is not required for coupling to Gs, but can serve as a binding site for "accessory" proteins (Zezula and Freissmuth, 2008). The sequence identity between the human A_1 and A_3 ARs is 49%, and the human A_{2A} and A_{2B} ARs are 59% identical. Particular conserved residues point to specific functions. For example, there are two characteristic His residues in TMs 6 and 7 of the A_1, A_{2A} and A_{2B} ARs. In the A_3 AR, the His residue in TM6 is lacking but another His residue has appeared at a new location in TM3. All of these His residues have been indicated by mutagenesis to be important in the recognition and/or activation function of the receptors (Costanzi et al., 2007; Kim et al., 2003). Similar to the function and regulation of others GPCRs, both activation and desensitization of the ARs occur after agonist binding. Interaction of the activated ARs with the G proteins leads to second messenger generation and classical physiological responses. Interaction of the activated ARs with G protein-coupled receptor kinases (GRKs) leads to their phosphorylation. ARs responses desensitize rapidly, and this phenomenon is associated with receptor downregulation, internalization and degradation (Klaasse et al., 2008). The most rapid downregulation among the AR subtypes is generally seen with the A_3 AR, due to phosphorylation by GRKs. The A_{2A} AR is only slowly desensitized and internalized as a result of agonist binding.

A_1 adenosine receptors

A_1 ARs have been cloned from several species. The human A_1 AR subtype gene (ADORA1) has been localized to chromosome 1q32.1 (Townsend-Nicholson et al., 1995). The variability in the primary sequence of the A_1 AR between species is less than 10% for A_1 from dog, rat and cow and less than 5% between bovine and human A_1 AR (Tucker and Linden, 1993). The A_1 ARs is widely express throughout the body, having its highest expression in the brain, spinal cord, atria and adipose tissue (Baraldi et al., 2000). Adenosine via A_1 ARs reduces heart rate, glomerular filtration rate, and renin release in the kidney; it induces bronchocostriction and inhibits lipolysis (Elzein and Zablocki, 2008). The most

widely recognized signalling pathway of A_1 ARs is inhibition of adenylate cyclase causing a decrease in the second messenger cAMP (Fredholm et al., 2001). This in turn modulates the activity of cAMP-dependent protein kinase, which phosphorilates diverse protein targets. Another signalling mechanism of A_1 ARs is activation of phospholipase C (PLC) leading to membrane phosphoinositide metabolism and increased production of inositol 1,4,5-triphosphate (IP_3) and Ca^{2+} mobilization. Elevation of cytosolic Ca^{2+} by IP_3 can stimulate a variety of signalling pathways, including protein kinase C (PKC), phospholipase A_2 (PLA_2), Ca^{2+}-dependent K^+ channels and nitric oxide synthase (NOS). Depletion of Ca^{2+} from IP_3-sensitive pools may promote Ca^{2+} influx from extracellular sources. A_1 AR display two different affinities for agonist, which have classically been attributed to a different coupling to heterotrimeric G proteins. According to this two independent site model, CGRP complex display high affinity for agonist and uncoupled receptors display low affinity. The reported cluster-arranged cooperative model predicts thet the high- and low-affinity sites are a consequence of the negative cooperativity of agonist binding and do not seem to be related to the content of G protein-coupled or –uncoupled receptors (Franco et al., 1996). In the early 1990s Bruns et co-workers reported on various allosteric modulators, 2-amino-3-benzoylthiphene derivatives capable of enhancing the binding and activity of reference A_1 receptor agonists, such as N^6-cyclopentyladenosine (CPA) (Bruns et al., 1990a, Bruns et al., 1990b). It was demonstrated that 2-amino-4,5-dimethylthien-3-yl)[3-(trifluoromethyl)phenyl]-methanone (PD 81,723) represents a specific and selective allosteric enhancer of agonist binding to the A_1 ARs originally identified from a screening library of benzodiazepine-like compounds. PD 81,723 has been shown to enhance agonist binding and the functional activation of the A_1 ARs in both brain and cardiovascular tissues (Amoah-Apraku et al., 1993, Bhattacharya et al., 1995, 1996; Janusz et al., 1991, 1993, Mudumbi et al., 1993). The available data indicate that PD 81,723 functions to stabilize a high affinity or agonist-preferring state of the A_1 ARs (Bruns et al., 1990b, Bruns, 1996, Linden et al., 1997). This hypothesis is supported by data demonstrating that the allosteric actions of PD 81,723 can be greatly enhanced in the presence of GTP (Bruns et al., 1990b) and that PD 81,723 can significantly slow the dissociation kinetics of agonist binding from rat brain membranes (Bruns et al., 1990b) and from the recombinant human A_1 ARs (Bhattacharya et al., 1995,

1996). Thus, the putative mechanism by which PD 81,723 enhances the activation of A_1 ARs is thought to be via an allosterically mediated conformational shift of the receptor to a high affinity state thereby improving receptor/effector coupling (Bruns et al., 1990b). The binding site of PD 81,723 on the adenosine A_1 ARs has yet to be elucidated.

A_{2A} adenosine receptors

The A_{2A} AR has been cloned from several species such as human, rat, mouse and canine. The gene for A_{2A} AR has been mapped to human chromosome 22 with reported chromosomal localizations of 22q11.2 (Peterfreund et al., 1996). The A_{2A} ARs is present in a wide variety of organs including major peripheral tissue (e.g. liver, heart, lung and the immune system) and the central nervous system (CNS) (Lee et al., 2003). The most commonly recognized signal transduction mechanism for A_{2A} ARs is activation of adenylate cyclase. This implies primarly coupling with the Gs, although other G proteins may also be involved. In striatum the A_{2A} ARs interacts with Golf proteins (Corvol et al., 2001). It is not known if there are significant differences in receptor affinity or in signalling closely dependent by the interaction with the specific G proteins. In addition other G protein pathways could be implicated, in the regulation of processes, for example via phosphorylation. There is no compelling reason to assume that this GPCR coupling to members of the Gs family would signal in anything but a canonical way. Thus, most effects are probably due to activation of adenylyl cyclase and generation of cAMP. The A_{2A} ARs can recruit β-arrestin via a GRK-2 dependent mechanism (Khoa et al., 2006). This is influenced by activation of cytokine receptors, which cause reduced desensitization of the A_{2A} ARs (Khoa et al., 2006). One key target of PKA is the cAMP responsive element-binding protein (CREB) which is critical for many forms of neuronal plasticity as well as other neuronal functions (Josselyn and Nguyen, 2005). Phosphorilation of CREB by PKA activates CREB and turns on genes with cAMP responsive elements (CRE sites) in their promoters. One important feature of CREB is that it is a point of convergence for the cAMP/PKA and MAPK pathways. Stimulation of the A_{2A} ARs counteracts the inhibition of neurite outgrowth due to MAPK blockade. Stimulation of the A_{2A} ARs alone also activates the Ras/Raf-1/MEK/ERK signalling through PKA-dependent and PKA-independent pathways via Src- and

Sos- mediated mechanism, respectively (Schulte and Fredholm, 2003). Phosphorylated CREB was therefore proposed to mediate the anti-inflammatory effect of the A_{2A} ARs and inhibition of NFκB by A_{2A} ARs activation during acute inflammation in vivo was demonstrated (Fredholm et al., 2007). Activation of a multiple signalling pathways by the A_{2A} ARs appears to contribute to its diverse and complex functions in various tissues.

A_{2B} adenosine receptors

A_{2B} AR mRNA was originally detected in a limited number of rat tissues by Northern blot analysis, with the highest levels found in cecum, bowel, and bladder, followed by brain, spinal cord, lung, epididymis, vas deferens, and pituitary. The use of more sensitive reverse transcriptase-polymerase chain reaction techniques revealed a ubiquitous distribution of A_{2B} ARs (Spicuzza et al., 2006). mRNA encoding A_{2B} AR was detected at various levels in all rat tissues studied, with the highest levels in the proximal colon and lowest in the liver. In situ hybridization of A_{2B} ARs showed widespread and uniform distribution of A_{2B} AR mRNA throughout the brain (Dixon et al., 1996). The human A_{2B} ARs gene (ADORA2B) has been localized to chromosome 1q32 (Jacobson et al., 1995b). Pharmacological identification of A_{2B} ARs, based on their low affinity and characteristic order of potency for agonists, also indicates a widespread distribution of A_{2B} ARs. Functional A_{2B} ARs have been found in fibroblasts and various vascular beds, hematopoietic cells, mast cells, myocardial cells, intestinal epithelial and muscle cells, retinal pigment epithelium, endothelium, and neurosecretory (Gessi et al., 2005). Although activation of adenylyl cyclase is arguably an important signaling mechanism for A_{2A} ARs, this is not necessarily the case for A_{2B} ARs, as other intracellular signaling pathways have been found to be functionally coupled to these receptors in addition to adenylyl cyclase. In fact activation of A_{2B} ARs can increase phospholipase C in human mast cells and in mouse bone marrow-derived mast cells. A_{2B} ARs activation also elevates IP_3 levels, indicating this receptor can couple also to Gq-proteins. A_{2B} ARs have been implicated in the regulation of mast cell secretion and, gene expression, intestinal function, neurosecretion, vascular tone and in particular asthma (Varani et al., 2005).

A_3 adenosine receptors

A_3 AR was identified relatively late in the history of ARs with the cloning, expression and functional characterization of a novel adenosine receptor from rat striatum (Zhou et al., 1992). The human A_3 AR has been localized to chromosome 1p13.3 (Monitto et al., 1995). The A_3 ARs has widely distributed its mRNA being expressed in testis, lung, kidney, placenta, heart, brain, spleen, liver, uterus, bladder, proximal colon and eye of rat, sheep and humans. However, marked differences exist in expression levels within and among species. In particular rat testis and mast cells express high concentrations of A_3 ARs mRNA, while low levels have been detected in most other rat tissues (Gessi et al., 2008). Lung and liver have been found as the organs expressing high levels of A_3 ARs mRNA in human, while low levels have been found in aorta and brain. The classical pathways associated with A_3 ARs activation are the inhibition of adenylyl cyclase activity, through the coupling with Gi proteins, and the stimulation of PLC, IP_3 and intracellular Ca^{2+}, via Gq proteins (Fredholm et al., 2001). However, more recently additional intracellular pathways have been described as relevant for A_3 ARs signaling. For example, in the heart, A_3 AR mediates cardioprotective effects through ATP-sensitive potassium (KATP) channel activation. Moreover, it is coupled to activation of RhoA and a subsequent stimulation of phospholipase D (PLD), which in turn mediates protection of cardiac myocytes from ischemia (Mozzicato et al., 2004). In addition, in different recombinant and native cell lines, A_3 ARs is involved, like the other adenosine subtypes, in the modulation of mitogen-activated protein kinase (MAPK) activity (Schulte and Fredholm, 2003). A_3 ARs signaling in Chinese Hamster Ovary cells transfected with human A_3 ARs leads to stimulation of extracellular signal-regulated kinases (ERK1/2). In particular, A_3 ARs signaling to ERK1/2 depends on $\beta\gamma$ release from pertussis toxin (PTX)-sensitive G proteins, phosphoinositide 3-kinase (PI3K), Ras and mitogen-activated protein kinase kinase (Schulte and Fredholm, 2003). It has been reported that A_3 ARs activation is able to decrease the levels of PKA, a downstream effector of cAMP, and of the phosphorylated form of PKB/Akt in melanoma cells. This implies the deregulation of the Wnt signaling pathway, generally active during embryogenesis and tumorigenesis to increase cell cycle progression and cell proliferation (Fishman et al., 2002). Involvement of the PI3K/PKB pathway

has been linked with preconditioning effects induced by A_3 ARs activation in cardiomyocytes from newborn rats (Germack and Dickenson, 2005).

ADENOSINE TRIPHOSPHATE (ATP)

Adenosine triphosphate or ATP is a ribonucleotide which is the main donor of energy in cellular reactions and is involved in the production of mechanical work in the active transport of ions and molecules and the synthesis of macromolecules. ATP consist af adenosine (composed of an adenine ring and a ribose sugar) and three phosphate groups connected with anhydride bonds (Figure 3). The energy content of an isolated molecule of ATP is a consequence of these anhydride bonds when they hydrolyzed, releases a large amount of energy.

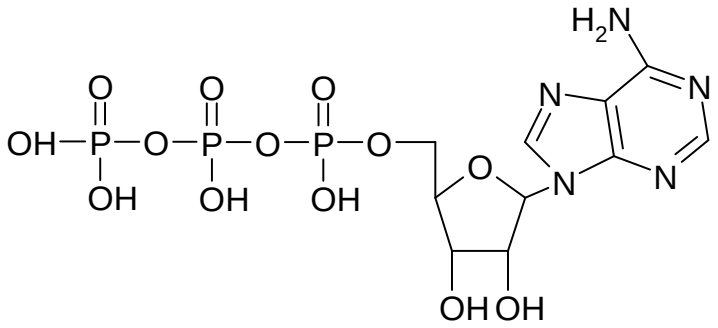

Figure 3: chemical structure of ATP

The purine and pyrimidine nucleotides are compartimentalized in the cell cytoplasm, where there are involved in the synthesis of nucleic acid, or in the cellular metabolism, two key functions, from which depends the survival of any cell. Only recently ATP is recognized as an extracellular messenger (Burstock, 2009). There are many studies in the literature indicating that ATP could function as a neurotransmitter. Infact it is released by the purinergic terminals and then recognized by membrane receptors (Ralevic and Burnstock, 1998; Boeynaems et al., 2005). Investigations conducted in the nervous system clarifies the role of extracellular nucleotides in different anatomical districts, as regulators of proliferation and cell differentiation, cytokine release, chemotaxis, the formation of reactive oxygen and nitrogen species, secretion of lysosomal factors, as well as

induction of apoptosis or cell necrosis (Burnstock, 2007). ATP and other nucleotides are taken up by and stored in secretory and synaptic vescicles. As revealed recently, accumulation of ATP into vescicular can be mediated by a Cl^--dependent vesicular nucleotide transporter (VNUT), which belongs to the SLC17 anion transporter family, which includes also vesicular glutamate transporters (Sawada et al., 2008). VNUT preferentially recognizes ATP, GTP and ADP and is inhibited by diATP. ATP is probably present in every synaptic and/or secretory vesicle, although at different concentrations, and can be co-stored and co-released with other neurotransmitters (γ-aminobutyric acid, noradrenaline or glutamate). At present, it is unclear whether various physiological nucleotide-receptor agonists (ATP, ADP, UTP, UDP, UDP and NAD^+) are released by common mechanisms. There is compelling evidence for exocytotic neuronal vesicular release of ATP (Pankratov et al., 2007) and recent studies also support a vescicular release of ATP from astrocytes, which perhaps involves lysosomes (Zhang et al., 2007). Evidence has been provided for additional mechanisms of nucleotide release, including ATP-binding cassette transporters, connexin or pannexin hemichannels, plasmalemmal voltage-dependent anion channels, in addition to $P2X_7$ receptors (Pankratov et al., 2006; Dubyak, 2006; Scemes et al., 2007). After release, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases, which is functionally important because ATP metabolites act as physiological ligands for various P2 purinergic receptors.

P2 Purinergic Receptors

Implicit in the concept of purinergic neurotransmission was the existence of postjunctional purinergic receptors, and the potent actions of extracellular ATP on many different cell types also implicated membrane receptors. The cytosolic concentration of ATP in mammalian cells is quite high (5-10 mM), but it increase within specialized structures for its accumulation, where ATP can reach values of over 100 mM. In extracellular fluids, however, the concentration of ATP is maintained at very low levels, in the order of 5-20 μM due to the presence of enzymes that hydrolyze (Burstock, 2007). Extracellular ATP interacts with the cell in two ways: at nanomolar and micromolar concentrations exerts its effects after the binding and activation of membrane receptors that mediate a fast response and a short distance. The action of these receptors occurs in a time

estimated to milliseconds. In 1994 Abbracchio and Burnstock (Abbracchio and Burnstock, 1994) on the basis of molecular structure and transduction mechanisms, proposed that purinoceptors should belong to two major families: a P2X family of ligand-gated ion channel receptors and a P2Y family of GPCRs.This nomenclature has been widely adopted and currently seven P2X subtypes and eight P2Y receptor subtypes are recognized, including receptors that are sensitive to pyrimidines as well as purines (Ralevic and Burnstock, 1998; North, 2002; Abbracchio et al., 2006). It is widely recognized that purinergic signalling is a primitive system involved in many non-neuronal as well as neuronal mechanisms, including exocrine and endocrine secretion, immune responses, inflammation, pain, platelet aggregation and endothelial-mediated vasodilatation (Burnstock and Knight, 2004; Burnstock 2006). Cell proliferation, differentiation and death that occur in development and regeneration are also mediated by purinergic receptors (Abbracchio and Burnstock, 1998; Burnstock 2002).

P2Y Purinergic Receptors

Recent biochemical investigations have shown that receptors similar to the P2Y group, appeared very early during evolution, supporting the hypothesis that purinergic and pirimidinergic signaling pathways are among the most ancient mechanisms of intercellular communication. The P2Y receptors are metabotropic GPCRs, characterized by a typical seven-transmembrane helices: the N-terminal domain facing the extracellular side, while the C-terminal domain protudes into the cell cytoplasm interacting with G proteins to mediated the activation of intracellular pathways (Von Kügelgen, 2006). The first P2Y receptor was cloned in 1993, since then, numerous other family members were isolated and cloned. At the present, there are eight accept human P2Y receptors ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) that have preferential affinity for ATP and related nucleotides. The missing numbers represent either non-mammalian orthologs, or receptors having some sequence homology to P2Y receptors, but for which there is no functional evidence of responsiveness to nucleotides. P2Y receptors differ from other families of GPCRs for their high heterogeneity in the amino acid sequence. Several studies have shown that these receptors are suitable for a neuromodulating function because they may perceive low levels of ATP (Abbracchio et al., 2006).

The binding to the receptors by extracellular nucleotides determines the activation of G proteins, phospholipase C (PLC) with subsequent formation of inositol phosphate 3 (IP_3) which induces the release of Ca^{2+} from intracellular stores, and diacylglycerol (DAG), that activates the protein kinase C (PKC). Activation of adenylyl cyclase generates cAMP, which activates protein kinase A (PKA). The increasing cytosolic concentration of Ca^{2+} and the activation of PKA and PKC mediate the cellular responses due to activation of P2Y receptors. The complexity of the intracellular pathways of P2Y receptors translates into a wide-ranging effect on many cellular functions such as proliferation, apoptosis, metabolism, secretory activity and cell migration. It has been demonstrated that each P2Y receptor subtype is associated with one or more G proteins subfamily (Gs, Gi/o, $Gq_{/11}$, $G_{12/13}$). For example, $P2Y_2$ subtype, is coupled to Gi, Go, $G_{q/11}$ or G_{12}. It remains to clarify which mechanisms allow each P2Y subtype to associate to different G proteins and generate multiple pathways of signal transduction. A possible explanation might be that many of P2Y receptors have a binding specificity for more than one nucleotide, and it is possible that the binding of different nucleotides, on the same receptor, induces distinct conformational states that can interact with different G proteins (Abbracchio et al., 2006). For example, ADP is the preferential agonist of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$, ATP and UTP are equipotent in the activation of $P2Y_2$, whereas UTP and UDP are preferential agonists of $P2Y_4$ and $P2Y_6$, respectively; $P2Y_{11}$ is activated only by ATP. The last cloned P2Y receptor, $P2Y_{14}$, has a pharmacological behaviour different from the other P2Y because it binds UDP-glucose as agonist (Chambers et al., 2000; Abbracchio et al., 2003). Mutagenesis study of P2Y receptors have demonstrated the importance of amino acid residues present in transmembrane regions 6 and 7 to determining the power and specificity of the agonist (Erb et al., 1995). P2Y receptors have been identified in several cells tipes such as epithelial cells, endothelial cells, striated and smooth muscle cells, neurons, fibroblasts, monocytes, macrophages, neutrophils, eosinophils and activated T lymphocytes (Abbracchio et al., 2006). $P2Y_1$ was identified in platelets, heart, connective tissue, immune system and nervous tissue. At the level of platelets plays an important role in the aggregation, where the absence of this receptor prolongs bleeding time and protects against thromboembolism ADP-dependent (Fabre et al., 1999). The expression of $P2Y_1$ and $P2Y_2$ in smooth muscle cells and vascular

endothelium induces vasodilatation (Di Virgilio et al., 2001). The $P2Y_4$ receptor is expressed in the placenta, while it is slightly expressed in the vascular system. The $P2Y_6$ receptor is expressed in the lung, heart, aorta, spleen, thymus, placenta and brain. The $P2Y_{11}$ receptor is expressed in spleen, intestine, in the myocardium, in dendritic cells and macrophages (Abbracchio et al., 2006). It is also expressed in several cell lines such as HL-60, in which induces granulocyte differentiation (Communi et al., 2000a, van der Weyden et al., 2000). $P2Y_{12}$ is expressed abundantly in platelets, lymphocytes and to a lesser degree in the brain, whereas $P2Y_{13}$ is found predominantly in spleen and brain, but also in lymphnodes and bone. The $P2Y_{14}$ receptor is mainly expressed in placenta, adipose tissue, stomach and intestine, and at low levels of it is detected in brain, spleen, lung and heart (Abbracchio et al., 2006). The activation of this receptor mediates chemotaxis in a population of hematopoietic cells of bone marrow (Lee et al., 2003).

P2X Purinergic Receptors

P2X receptors are ionotropic receptors, ie receptors whose activation causes the direct opening (or closure) of selective membrane channels for mono- and bivalent cations, capable of determining the leakage of K^+ and the entry of Na^+ and Ca^{2+} in the cell. The first cDNAs encoding P2X receptor subunits were isolated in 1994. Members of the family of ionotropic $P2X_{1–7}$ receptors show a subunit topology of intracellular N- and C-termini possessing consensus binding motifs for protein kinases; two transmembrane spanning regions (TM1 and TM2), the first involved with channel gating and the second lining the ion pore; a large extracellular loop, with 10 conserved cysteine residues forming a series of disulfide bridges; a hydrophobic H5 region close to the pore vestibule, for possible receptor/channel modulation by cations and anATP-binding site, which may involve regions of the extracellular loop adjacent to TM1 and TM2. The $P2X_{1–7}$ receptors show 30– 50% sequence identity at the peptide level (North 2002; Stojilkovic et al., 2005; Egan et al., 2006; Roberts et al., 2006). The stoichiometry of $P2X_{1–7}$ receptors is thought to involve three subunits, which form a stretched trimer or a hexamer of conjoined trimers (North 2002; Nicke et al., 1998). The pharmacology of the recombinant P2X receptor subtypes expressed in oocytes or other cell types is often different from the pharmacology of P2X-

mediated responses in naturally occurring sites (Gever et al., 2006). Several contributing factors may account for these differences. First, heteromultimers as well as homomultimers are involved in forming the trimer ion pore (Nicke et al., 1998). For example, heteromultimers are clearly established for $P2X_{2/3}$ receptors. $P2X_{1/2}$, $P2X_{1/5}$, $P2X_{2/6}$, $P2X_{4/6}$ and more recently $P2X_{1/4}$ heteromultimers have also been identified. $P2X_7$ does not form heteromultimers, and $P2X_6$ will not form a functional homomultimer. Second, spliced variants of P2X receptor subtypes might play a part. For example, a splice variant of $P2X_4$ receptor, while it is nonfunctional on its own, can potentiate the actions of ATP through the full-length $P2X_4$ receptors (Townsend-Nicholson et al., 1999). The P2X subunit proteins are 384 ($P2X_4$) to 595 ($P2X_7$) amino acids long (Roberts et al., 2006). Each has two hydrophobic regions. All the P2X receptor subunits have consensus sequences for N-linked glycosylation. There are seven genes for P2X receptor subunits. $P2X_4$ and $P2X_7$ subunit genes are located close to the tip of the long arm of chromosome 12 (North 2002). $P2X_4$ and $P2X_7$ subunits are among the most closely related pairs in amino acid sequences. $P2X_1$ and $P2X_5$ genes are also very close together on the short arm of chromosome 13. The remaining genes are on different chromosomes. Several full-length non-mammalian vertebrate sequences have been identified. There are no reports of homologous sequences from invertebrate species, although there is considerable functional evidence that extracellular ATP and other nucleotides can directly gate ion channels in invertebrates (Egan et al., 2006; Roberts et al., 2006). Following the binding with the ATP, the P2X receptor channel opens, generating a movement of cations across the cell membrane. This movement of ions produces changes in electrical potential of the cell, with membrane depolarization which, in turn, activates calcium-channel voltage-dependent. The accumulation of calcium ions in the cytoplasm is also responsible of the activation of numerous signal transduction pathways through the activation of MAPKs, PKC and calmodulin (Communi et al., 2000b; North, 2002). The extent of cellular responses induced by these receptors, in addition to being modulated by transmembrane potential difference, is also regulated by the duration of receptor stimulation. Continuous stimulation of the receptor leads to a state of desensitization, in which the receptor no longer responds to the endogenous agonist. The rate with which this process is established, and involves all the molecules of the receptor, is finely regulated by

the cell through specific phosphorylation induced by second messengers. For example, $P2X_1$ and $P2X_3$ receptors have a rapid desensitation when continuously stimulated with ATP and $P2X_2$ receptor channel stays open until the ATP interacts with it. Receptor activity and intensity of the response is also strongly modulated by extracellular pH and the presence of heavy metals (zinc and cadmium) that act as allosteric modulators (Huidobro-Toro et al., 2008). For example, the sensitivity of $P2X_1$, $P2X_3$ and $P2X_4$ receptors to ATP is attenuated when the extracellular pH is greater than seven, while the affinity of the $P2X_2$ receptor in these conditions, significantly increases. Zinc, however, increases the ionic current through $P2X_2$, $P2X_3$ and $P2X_4$ receptors while it inhibits the ionic current through $P2X_1$ receptor (Gever et al., 2006). The ability of pH and metal allosteric modulators of these receptors appears to be attributed to the presence of histidine residues in the extracellular region (North, 2002). These receptors are generally expressed by excitable cells like neurons, smooth and striated muscle cells. P2X receptors are involved in fast synaptic interneuronal transmission in CNS or in muscle contraction. They are also present at the level of immune system cells such as monocytes, macrophages, dendritic cells, thymocytes and other lymphoid cells, in which seem to mediate multiple responses including apoptotic cell death (Pizzo et al., 1991; Chvatchko et al., 1996). $P2X_1$ receptors are expressed predominantly in human smooth muscle cells of the deferens vessel and of the bladder as well as in brain, heart and platelets. While $P2X_2$ receptors are present in sensory ganglia, brain, chromaffin cells, in the retina, cochlea and in smooth muscle. $P2X_3$ receptors are found in sensory fibers in the brain and heart. Experiments in vivo have been attributed to polymer $P2X_{2/3}$ a role in signal transmission in chronic inflammatory pain and neuropathic pain (Barclay et al., 2002; Wirkner et al., 2007). $P2X_4$ receptors in addition to being expressed at high levels in the brain, are localized at the level of colon, heart, epithelia and smooth muscle. In the rat this receptors was detected in the hippocampus, the brain, in dorsal ganglion (DRG) and endocrine tissue. $P2X_5$ receptors are localized in the brain, heart, spine, lymphocytes and thymus. In humans, $P2X_6$ receptors are present in the brain, sensory and autonomous ganglia. The expression of $P2X_7$ receptors are high in immune cells such as monocytes, macrophages, microglia, dendritic cells (North, 2002; Burnstock, 2007; Surprenant and North, 2008). In literature, numerous studies have attempted to define the pharmacological profile of agonists

and antagonists of P2X receptors (Burnstock, 2007). Indeed there are many works reporting the effect of agonists and/or antagonists of the purinergic system in different tissues and/or organs. These results show sometimes inconsistency on the results obtained, this is probably due to the low affinity and selectivity of the ligands used and to the ubiquitous presence of several purinergic subtypes. To date, there are numerous agonists and/or antagonists for the purinergic receptors even though most of them are not selective.

$P2X_1$ and $P2X_3$ receptors

$P2X_1$ and $P2X_3$ receptors are transmembrane channel permeable to cations (Na^+, Ca^{2+} and K^+) which represent respectively the first and the third member of the P2X family. Exposure to ATP or selective agonists activates these receptors transiently changing the channel conformation. Opening of these receptors generates a movement of cations across the cell membrane that produces changes in the electrical potential of the cell, with consequent membrane depolarization, which in turn, activates calcium-channel voltage-dependent (Surprenant and North, 2008). $P2X_1$ purinergic receptors have been cloned and expressed in different animal species such as rat, mice and human. The human gene coding for the purinergic receptors $P2X_1$ is mapped in 17p13.2 and shows characteristics very similar to that of rat (North, 2002). $P2X_1$ receptors are proteins consisting of 399 amino acids and has a molecular mass of 70 kD, almost double that provided by the amino acid sequence, probably because of glycosylation of the extracellular domain (Sun et al., 1998). $P2X_1$ subunits have four potential glycosylation sites (Asn153, Asn184, Asn284, Asn300 in rat) (Nicke et al., 1998) whose deletion leads to inactivation of the ion channel (Newbolt et al., 1998, Torres et al., 1998). The human $P2X_1$ receptor is expressed not only in certain brain areas, but mainly in peripheral vascular smooth muscle cells of the vas deferens, arteries, arterioles, bladder and bowel, and heart in platelets (Gever et al., 2006). At the level of smooth muscle, activation of this receptor by agonist leads to contraction. Studies in $P2X_1$ knock-out mice have allowed to demonstrate that the absence of this receptor results in a reduced contraction of the vas deferens, male infertility, reduced vasoconstriction to nerve stimulation, reduced autoregolating blood flow in the kidney (Mulryan et al., 2000; Vial et al., 2000). $P2X_1$ receptors are expressed at high concentration also in platelets, where their activation by ATP is partly involved in the regulation of various platelet function. The importance of

this receptor in promoting and maintaing the platelet activation was confirmed by knock-out mouse in which the lack of $P2X_1$ receptor expression is associated with changes in aggregation, secretion and platelet adhesion and reduction of thrombus growth (Hechler et al., 2003). Even $P2Y_1$ and $P2Y_{12}$ receptors are abundantly expressed in platelets and involved in their regulation (Fabre et al., 1999). With immunohistochemical studies, it was shown that $P2X_1$ receptors are expressed, even if in low concentrations, in lymphocytes (Wiley et al., 1996) and in heart muscle (Dhulipala et al., 1998). $P2X_3$ purinergic receptors have been isolated from sensory neurons of dorsal root ganglia (DRG) of rat and human heart (Chen et al., 1995, Lewis et al., 1995, Garcia-Guzman et al., 1997). The human gene coding for them map 11q12 and the percentage of identity between the sequences of human and rat $P2X_3$ is high. (North and Surprenant, 2000; North, 2002). $P2X_3$ receptors are composed of 397 amino acids and as $P2X_1$ receptors have four potential glycosylation sites. Furthermore, both $P2X_1$ that $P2X_3$ subunits assemble as trimers (Nicke et al., 1998). $P2X_3$ and $P2X_{2/3}$ receptors are expressed predominantly in the major afferent neurons, ie in small diameter nociceptive sensory neurons of dorsal root and trigeminal ganglia and nodose (Thomas et al., 1998; Virginio et al., 1998; Cockayne et al., 2005). The nociceptive afferents are widely distributed throughout the body and they include both Aδ myelinated fibers of medium diameter, both the C fibers of small diameter, non-myelinated slow conductance (Kirkup et al., 1999; Cockayne et al., 2000; Lee et al., 2000; Brouns et al., 2000; Wynn et al., 2004). At the central level $P2X_3$ and $P2X_{2/3}$ are present in the dorsal horn and in the nucleus of the solitary tract (NST) which appear to be involved in the modulation of presynaptic glutamate release (Gu et al., 1997; Hechler et al., 2003; Gever et al., 2006). Studies have shown that $P2X_3$ and $P2X_{2/3}$ are expressed in the enteric nervous system (Poole et al., 2002) and in several epithelial tissues where responses appear to modulate some chemical or mechanical (Fu et al., 2004). In vivo experiments have allowed to attribute to $P2X_3$ and $P2X_{2/3}$ an important role in the signaling of chronic inflammatory and neuropathic pain (Barclay et al., 2002). Studies in mice $P2X_3$ knock-out showed that the absence of this receptor results in reduced urinary and abnormal reflex response to pain stimuli (Cockayne et al., 2000; Gever et al., 2006). Antagonists for this receptor and its heteromeric form could therefore have a therapeutic role for the treatment of chronic pain (Wirkner et al., 2007). $P2X_3$ receptors are

pharmacologically similar to $P2X_1$ receptors, both rapidly desensitization when exposed to high concentrations of ATP. It was noted that the time of desensitization for the $P2X_1$ receptor is approximately 300 milliseconds while for the $P2X_3$ receptor is about 100 milliseconds. The ability for the $P2X_1$ receptor to function again is achieved after 10-30 min and for $P2X_3$ receptor is 15 min (North et al., 2000). It has been shown that both receptor subtypes are activated to ATP and to α,β-meATP (pEC_{50}> 6) and inhibited not only by Suramin but also by TNP-ATP (pIC_{50}> 8). In literature, studies have defined the pharmacological profile of agonists and antagonists specific for $P2X_1$ and $P2X_3$ receptors. Selective agonists for the $P2X_1$ receptor were analog of ATP as γβ-MeATP (γ,β-methylene-ATP) and diadenosine polyphosphates (Ap4A) while $P2X_3$ receptor showed selectivity for diadenosine polyphosphates (Ap3A). Selective $P2X_1$ antagonist were Suramin analogues NF449 and NF864 dipeptides and the compound RO-1 while $P2X_3$ receptor shown affinity to tricarboxylic acid A317491 and RO-3 (composed of dipeptides) (Gever et al. , 2006). The activity and intensity of the response of these receptor subtypes is also strongly modulated by the presence of heavy metals such as zinc and extracellular pH that act as allosteric modulators. Recent studies have shown that the extracellular pH negatively regulates $P2X_1$ and $P2X_3$ receptors while zinc inhibits $P2X_1$ receptors and positively modulates $P2X_3$ receptors. It is well known that calcium influences these receptors by the inhibition of $P2X_3$ receptors even if no effect on $P2X_1$ has been reported (Gever et al., 2006).

ADENOSINE, ATP AND INFLAMMATION

Adenosine receptors on immune cells

Adenosine is an endogenous signalling molecule that engage cell surface ARs to regulate numerous physiological and pathological processes. Substantial evidence demonstrates that adenosine is an important signalling molecule and ARs are important molecular targets in the pathophysiology of inflammation. All inflammatory cells express ARs, and research into the consequences of ARs activation has identified numerous avenues for adenosine-based therapeutic intervention. Indeed , adenosine-based approaches are currently being developed

for the treatement of various disorders where inflammatory modulation is a key component.

Neutrophils

Neutrophils are the most abundant leukocyte and represent rhe body's first line of defence in response to a pathogenic challenge; they are the predominant leukocytes involved in acute inflammation. All four adenosine receptors subtypes are expressed on neutrophils. At submicromolar concentrations, A_1 ARs activation on human neutrophils produces proinflammatory response by promoting chemotaxis and adherence to the endothelium (Bours et al. 2006). Activation of A_{2A} ARs and A_{2B} ARs on neutrophils is anti-inflammatory. High concentration of adenosine inhibit neutrophils adhesion to endothelial cells by activating these receptors (Bours et al. 2006, Sullivan et al. 2004). In human neutrophils, A_{2A} ARs activation inhibits the formation of reactive oxygen species (Salmon and Cronstein 1990). Activation of A_{2A} ARs on neutrophils produced an anti-inflammatory effect by decreasing the formation of the proinflammatory cytochine TNF-α (Harada et al. 2000), chemokines such as macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, MIP-2α/CXCL2 and MIP-3α/CCL20 (McColl et al. 2006), and leukotriene LTB4 (Flamada et al. 2000, 2002), and platelet activating factor (PAF) (Flamada et al. 2006). Other important immunoregulatory effects mediated by the A_{2A} ARs include the inhibition of Fc gamma (Fcγ) receptor-mediated neutrophils phagocytosis and inhibition of degranulation (Bours et al. 2006, Sullivan et al. 1999). Activation of the A_{2B} ARs inhibits neutrophils extravasation across human umbilical vein endothelial cell (HUVEC) monolayers and inhibits the release of vascular endothelial growth factor (VEGF) (Wakai et al. 2001). Conflicting reports suggest that activation of A_3 ARs on neutrophils may produce proinflammatory or anti-inflammatory effects. Studies with A_3 ARs knockout mice suggest that A_3 ARs promotes recruitment of neutrophils to lungs during sepsis (Inoue et al. 2008). Moreover, A_3 ARs play an important role in the migration of neutrophils in response to chemoattractant molecules released by microbes (Chen et al. 2006). Selective A_3 ARs antagonists inhibit fMLP-mediated chemotaxis in neutrophils (Chen et al. 2006). In other studies, activation of A_3 ARs has been shown to counteract inflammation by inhibiting degranulation and oxidative burst (Gessi et al. 2002).

Monocytes and Macrophages

Monocytes and macrophages are an heterogenous group of mononuclear cells that present an early line of innate immune defense. They represent a primary source of inflammatory modulators and are highly adaptable with a phenotype that can change rapidly in response to the local environment of the inflamed tissue. Macrophages also show an important role in terminating the inflammatory process, which is critical for preventing excessive tissue injury. All four ARs are expressed on monocytes and macrophages, although expression levels differ markedly throughout the maturation and differentiation process. (Thiele et al. 2004). In human monocytes, A_1 ARs activation produces a proinflammatory effect whereas A_{2A} ARs activation produces an anti-inflattory effect. A key function of the A_1 ARs is a rapid enhancement of the activity of the Fcγ receptor (Salmon et al. 1993). Activation of A_{2A} ARs limits inflammatory reactions by inhibiting phagocytosis in monocytes (Salmon et al. 1993) and macrophages (Eppell et al. 1989), decreasing the production of reactive oxygen species (Thiele et al 2004) and altering cytokine release. In addition, A_3 ARs activation inhibits fMLP-triggered respiratory burst in monocytes (Broussas et al. 1999). Monocytes and macrophages are primary sources of TNF-α, a proinflammatory cytokine involved in the pathophysiology of a number of chronic inflammatory diseases. Early studies suggested that activation of A_{2A} ARs suppresses production of TNF-α in monocytes activated by bacterial lipopolysaccharide (LPS) (Le Vraux et al. 1993). In primary cultures of monocytes activated by LPS and LPS-stimulated mause macrophages, activation of the A_{2A} ARs attenuate the release of TNF-α, wherease activation of the A_1 ARs and A_3 ARs had no effect on the formation of TNF-α (Zhang et al. 2005; Ezeamuzie and Khan 2007). Similar results were obtained in studies with primary cultures of mouse peritoneal macrophages (Kreckler et al. 2006). In other studies, activation of A_3 ARs was shown to inhibit LPS-induced TNF-α release in vitro in the RAW 264.7 murine leukemia macrophage line (Martin et al. 2006), U937 human leukemic macrophage line (Sajjadi et al. 1996), murine J774.1 macrophages (McWhnney et al. 1996) and in vivo in endotoxemic mice (Hasko et al. 1996). In the RAW 264.7 macrophage line, the inhibitory effects of A_3 ARs were mediated by a mechanism involving Ca^{2+}-dependent activation of NF-κB (Martin et al. 2006).

Dendritic cells

Dendritic cells (DCs) are highly specialized antigen-presenting cells that play an important role in the initiation and regulation of immune responses by migrating to sites of injury and infection, processing antigens, and activating naïve T cells. Immature DCs (imDCs) undergo a maturation process following exposure to proinflammatory signals, including pathogens, LPS, TNF-α, IL-1 and IL-6. the maturation process results in decreased phagocytic activity and increased expression of membrane major histocompatibility complex (MHC). Mature DCs release a number of cytokines, including TNF-α, IL-12 and IL-10. ARs are differentially expressed on human DCs (Fossetta et al. 2003). Immature, undifferentiated human DCs express mRNAs for the A_1 AR, A_{2A} AR and A_3 AR but not for the A_{2B} AR (Fossetta et al. 2003). Activation of the A_1 ARs and A_3 ARs subtypes in undifferentiated DCs induces chemotaxis and mobilization of intracellular Ca^{2+}, while activation of the A_{2A} AR subtypes has no effect (Panther et al. 2001). Activation of A_1 ARs in resting DCs suppresses vescicular MHC class I cross-presentation by a Gi-mediated pathways (Chen et al. 2008). Following treatment with LPS to induce differentiation and maturation, human DCs primarily express the A_{2A} ARs (Panther et al. 2001; Fossetta et al. 2003). Activation of the A_{2A} ARs increases adenylyl cyclase activity and inhibits production of the proinflammatory cytokine IL-12, thereby reducing the ability of the DC to promote the differentiation of T cells o the Th-1 phenotype, and stimulates the production of the anti-inflammatory cytokine IL-10 (Panther et al. 2001, 2003). In mature DCs the A_{2A} ARs is the predominant subtype and A_{2A} ARs activation decreass the production of IL-6, IL-12 and TNF-α (Schnurr et al. 2004). Moreover IL-3-induced maturation of human DCs results in a downregulation of A_1 ARs and an upregulation of A_{2A} ARs (Schnurr et al. 2004). The mouse DC line XS-106 expresses functional A_{2A} ARs and A_3 ARs (Dickenson et al. 2003). A_{2A} ARs activation increases cAMP levels and p42/p44 MAPK phosphorylation, whereas activation of A_3 ARs inhibits cAMP accumulation and increases in p42/p44 MAPK phosphorylation. Functionally, the activation of both subtypes produces a partial inhibition of LPS-induced release of TNF-α.

Lymphocytes

Lymphocytes are critically involved in adaptive immunity. Adenosine regulates multiple physiologic processes and inflammatory actions on lymphocytes. In early studies, it was demonstrated in mixed human lymphocytes that R-PIA and low concentrations of adenosine (1-100 nM) inhibit cAMP accumulation via A_1 ARs mechanism, while high concentrations of adenosine (100 nM- 100 μM) stimulated cAMP via an A_{2A} ARs mechanism (Marone et al. 1986, 1992). $CD4^+$ and $CD8^+$ T lymphocytes express A_{2A} ARs, A_{2B} ARs and A_3 ARs (Gessi et al. 2004, 2005; Hoskin et al. 2008). In activated human $CD4^+$ and $CD8^+$ T lymphocytes, A_{2B} ARs expression is increased and its activation is linked to decreased IL-12 production. Activation of human $CD4^+$ T lymphocytes with phytohemagglutinin results in increases in A_3 ARs mRNA and protein levels that are accompanied by increased agonist potency (Gessi et al. 2004). A number of studies suggest that A_{2A} ARs engagement on CD4+ T lymphocytes results in an anti-inflammatory effects. In mouse $CD4^+$ T lymphocytes, A_{2A} ARs engagements inhibits T-cell receptor (TCR)-mediated production of IFN-γ (Lappas et al. 2005). TCR activation results in A_{2A} ARs mRNA upregulation, which functions as an anti-inflammatory mechanism for limiting T-cell activation and subsequent macrophage activation in inflamed tissues (Lappas et al. 2005). Activation of the A_{2A} ARs on $CD4^+$ T lymphocytes prevents myocardial ischemia-reperfusion injury by inhibiting the accumulation and activation of $CD4^+$ T cells in the reperfused heart (Yang et al 2006b). moreover, an anti-inflammatory role in chronic inflammation was demonstrated for the A_{2A} ARs in an in vivo murine model of inflammatory bowel disease, where activation of the A_{2A} ARs attenuated the production of IFN-γ, TNF-α and IL-4 in mesenteric T lymphocytes in a rabbit models of colitis (Odashima et al. 2005). The result of a study (Takahashi et al. 2007b) suggest that the anti-inflammatory effect of adenosine in lymphocytes is mediated by A_{2A} ARs and a proinflammatory effect is closely associated with A_1 ARs. As such, the net effect of adenosine on lymphocytes activated by IL-8 is a complex function due to the presence of ARs. In primary cultures of B lymphocytes, activation of B-cell antigen receptors results in the activation of NF-κB pathways (Minguet et al. 2005). Adenosine inhibits the NF-κB pathways by a mechanism related to increased of cAMP levels and activation of PKA. This study suggest that

adenosine-mediated signals represent an important step in mediating the activation of B lymphocytes. In activated human and mouse natural killer (NK) cells, adenosine inhibited the production of cytokines and chemokines (Raskovalova et al. 2006). In in vitro studies with lymphocytes derived from mouse spleen, A_1 ARs activation increased NK cells activity while A_{2A} ARs activation decreased NK cells activity (Priebe et al. 1990). In mouse LAK cells, the adenosine agonist CADO inhibited the cytotoxic activity and attenuated the production of IFN-γ, granulocyte macrophage colony-stimulating factor, TNF-α and MIP-1 α (Lokshin et al. 2006). Taken together, these results suggest that elevated adenosine levels in tumors may inhibit the tumoricidal effect of activated NK cells. In addition, recent studies have shown that adenosine exhibits anti-infllammatory activities by engagin A_{2A} ARs on regulatory cells (Deaglio et al. 2007).

ATP receptors on immune cells

Extracellular ATP is an important mediator of cell-to-cell interactions in the nervous, vascular and immune system. In pathologic conditions, as inflammation, ATP can highly increase, following its release by damage cells, elevated levels of ATP led to the activation of purinergic receptors (P2X and P2Y). The activation by ATP of inflammatory/immune cells leads to their release of some inflammatory mediators. The blockade of the purinergic-mediated activation of the inflammatory/immune cells might represent a useful tool to reduce the spreading of the inflammatory response.

Neutrophils

Neutrophils are the body's first line of defence against pathogens and are critical effectors in both innate and humoral immunity. ATP may contribute to the regulation of neutrophil function during inflammatory and immune responses Furthermore, neutrophils are capable of releasing both ATP and adenosine following inflammatory activation. The functions of neutrophils may thus be subject to autocrine and paracrine control by endogenous ATP and adenosine. Neutrophils have been shown to express $P2Y_{1,2,4,6,11,14}$ and $P2X_{1,4,5,7}$ receptor subtypes (Verghese et al., 1996; Jin et al., 1998; Mohanty et al., 2001; Moore et al., 2001b; Suh et al., 2001; Moore et al., 2003b; Chen et al., 2004; Meshki et al., 2004). P2 receptor density in neutrophils may be subtype-specific. As a model of

promyelocytic neutrophil progenitors, human leukemic HL-60 cells have been shown to weakly express $P2X_{1,5}$ moderately express $P2X_7$ and $P2Y_{1,11}$ and strongly express $P2Y_{2,4,6}$ receptor subtypes. Granulocytic differentiation of HL-60 cells was shown to induce upregulation of $P2X_5$ and $P2Y_{11}$ receptor subtypes, but downregulation of $P2X_7$ receptors (Adrian et al., 2000; Communi et al., 2000, 2001). ATP at high micromolar concentrations may contribute to the differentiation of HL-60 cells into neutrophil like cells via stimulation of $P2Y_{11}$ receptors (Communi et al., 2000, 2001). Although mRNA for the $P2X_7$ receptor has been detected in human polymorphonuclear neutrophils (PMN) (Chen et al., 2004), functional expression of this receptor subtype by neutrophils remains controversial. Using a mouse anti-human $P2X_7$ receptor monoclonal antibody, Gu and co-workers detected little expression of $P2X_7$ receptor protein on the surface of PMN. However, these authors reported the presence of large intracellular amounts of $P2X_7$ protein in PMN, and suggested that these might constitute an intracellular receptor reserve from which $P2X_7$ receptors may be recruited to the surface following cellular activation (Gu et al., 2000). Extracellular ATP has been shown to stimulate neutrophil adhesion to endothelial cells (Rounds et al., 1999). Up-regulation of endothelial adhesion molecules such as E-selectin near inflammatory sites allows circulating neutrophils to tether to the endothelium, which results in rolling of neutrophils. ATP at low millimolar concentrations has been shown to induce up-regulation of E-selectin through $P2X_7$ receptor-mediated activation of NF-κB (Goepfert et al., 2000). During rolling along the endothelium, neutrophils are primed by various chemoattractants and chemokines secreted by endothelial cells. Micromolar concentrations of ATP have been shown to induce a rapid up-regulation of Mac-1 in neutrophils (Akbar et al., 1997). Following firm adhesion, neutrophils extravasate by transmigrating through the vascular endothelium. Extracellular ATP may facilitate transmigration by increasing endothelial permeability via activation of P2Y receptors (Tanaka et al., 2004). Once extravasated, neutrophils migrate to sites of inflammation or tissue damage; a process which is mediated by a variety of chemokines and chemoattractants. Effects of extracellular ATP on neutrophil migration are equivocal. At micromolar ATP concentrations, neutrophil motility (chemotaxis and chemokinesis) has been shown either to be: (a) unaffected (Aziz & Zuzel, 2001), (b) inhibited (Elferink et al., 1992), or (c) promoted via stimulation of P2Y2

receptors (Kaneider et al., 2004). ATP may also indirectly affect neutrophil migration by modulating formation of the potent neutrophil chemoattractant leukotriene (LT)-B_4, which is formed from arachidonic acid (AA) through the 5-lipoxygenase (5-LO) pathway. High micromolar ATP concentrations were shown to inhibit the release of AA, whereas low micromolar concentrations stimulated AA release by activating neutrophil P2Y2 receptors (Xing et al., 1992). These findings suggest that ATP may exert a dual modulatory role on neutrophil migration during inflammation. At low micromolar concentrations, ATP may promote neutrophil accumulation via P2Y2 receptor activation, either directly by acting as a chemoattractant or indirectly by facilitating LTB_4 production. Moreover, since ATP has been shown to stimulate production of IL-8 by both eosinophils and astrocytes (John et al., 2001; Idzko et al., 2003), ATP-mediated stimulation of chemokine release by cells near sites of tissue damage may contribute to neutrophil recruitment towards these sites. Upon arriving at inflamed sites where ATP levels are the highest, neutrophil recruitment may be no longer affected, even inhibited by ATP, allowing the neutrophils to exert their bactericidal functions. The first step in the bactericidal function of neutrophils is phagocytosis of pathogens, which has been shown to be stimulated by low micromolar concentrations of both ATP and ADP via activation of Mac-1 (Miyabe et al., 2004). Through activation of P2Y2 receptors (Meshki et al., 2004), micromolar concentrations of extracellular ATP stimulate the degranulation of both primary (Meshki et al., 2004) and secondary (specific) granules (Aziz & Zuzel, 2001). P2Y2 receptor-mediated LTB_4 generation, which may subsequently enhance granule secretion in an autocrine manner, has been proposed as a mechanism for nucleotide-induced neutrophil degranulation during inflammation (Kannan, 2001, 2002, 2003). In addition to stimulating degranulation, extracellular ATP has been shown to contribute to the initiation of the oxidative burst. ATP appears to prime neutrophils for functional responses to various inflammatory mediators, as indicated by increased production of reactive oxygen species (ROS, O_{2-} and H_2O_2) (Tuluc et al., 2005). Extracellular ATP at millimolar concentrations may induce ROS production even in quiescent neutrophils via stimulation of $P2X_7$ receptors (Suh et al., 2001). Extracellular ATP at micromolar concentrations has been shown to delay neutrophil apoptosis in synergy with the neutrophil survival factor granulocyte macrophage colony-stimulating factor

(GM-CSF), thus extending the functional life span of neutrophils (Gasmi et al., 1996).

Monocytes and macrophages

Monocytes and macrophages express multiple P2 receptor subtypes, i.e., monocytes express $P2Y_{1,2,4,6,11,12,13}$ and $P2X_{1,4,5,7}$ receptors (Gu et al., 2000; Chessel et al., 2001; Mehta et al., 2001; Moore et al., 2001b;Warny et al., 2001; Aga et al., 2002; Into et al., 2002a; Zhang et al., 2002; Derks and Beaman, 2004; Sluyter et al., 2004; Wang et al., 2004; Kaufmann et al., 2005), and macrophages express the same receptor subtypes except for $P2Y_{13}$ (Bowler et al., 2003; Hanley et al., 2004; Coutinho-Silva et al., 2005). P2 receptor expression may depend on maturation stage, since up-regulated expression of $P2X_7$ as well as P2Y receptors was noted upon differentiation of monocytes into macrophages (Falzoni et al., 1995). P2 receptor expression may also depend on the nature of cellular activation, since $P2X_7$ receptor expression and function appears to be up-regulated following classical activation of monocytes and macrophages by IFNγ, TNFα or LPS (Lemaire and Leduc, 2003). Classical activation of monocytes and macrophages with IFNγ and LPS has been shown to induce downregulation of P2Y2 receptor expression. (Martin et al., 1997). In contrast, activation of rat alveolar macrophages by the cytokines IL-4 and IL-10 was shown to induce down-regulation of functional $P2X_7$ receptors (Lemaire and Leduc, 2003). Extracellular ATP and ADP have been shown to increase adhesiveness of human promonocytic U937 cells (Ventura and Thomopoulos, 1991, 1995), suggesting that endogenous nucleotides may contribute to the adhesion of inflammatory monocytes to the vascular endothelium. Interaction of selectins with their respective ligands slows down rapidly flowing monocytes (monocyte rolling), a process to which ATP may contribute by up-regulating E-selectin expression through $P2X_7$-mediated activation of NFκB (von Albertini et al., 1998; Goepfert et al., 2000). Stimulation of monocyte $P2X_7$ receptors has been shown to induce shedding of L-selectin from the surface of monocytes (Sluyter et al., 2004). This might indicate that ATP at high extracellular levels contributes to activation and transmigration of monocytes, as leukocytes shed L-selectin upon activation and during (trans-)migration. Indeed, ATP was shown to stimulate transmigration of murine monocytes through an endothelial cell monolayer (Goepfert et al., 2001).

Subsequent to transmigration, monocytes migrate to inflammatory foci in response to various chemoattractants. Both ATP and ADP at micromolar concentrations have been shown to stimulate chemotaxis of murine monocytes as well as rat microglial cells (McCloskey et al., 1999; Goepfert et al., 2001; Honda et al., 2001). Similar levels of extracellular ATP were also shown to stimulate chemokinesis (i.e. undirected cell motility) of human monocytes (Kaufmann et al., 2005). Although the mechanism by which extracellular nucleotides mediate these chemotactic effects have not been clarified to date, stimulation of monocyte migration could be P2 receptor-mediated. Production of soluble immunomediators such as cytokines, chemokines and eicosanoids is essential in immunity and involves the activation of several transcription factors, including NFκB and activator protein AP-1. NFκB is activated in monocytes and macrophages by stimulation of $P2X_7$ receptors Many studies have confirmed that ATP at millimolar concentrations stimulates production of IL-1α, IL-1β IL-6, IL-18 (Mehta et al., 2001; Muhl et al., 2003; Sluyter et al., 2004) and TNFα (Guerra et al., 2003). These stimulatory effects are likely mediated through $P2X_7$ receptor activation. IL-1β is a potent pro-inflammatory cytokine mediating acute inflammatory responses. Mechanisms involved in ATP-mediated stimulation of IL-1β release have been extensively studied. Thus, extracellular ATP appears to be a crucial signal that triggers the synthesis and release of mature IL-1β following monocytes and macrophages priming by an inflammatory signal such as LPS. Binding of ATP to $P2X_7$ receptors causes opening of the channel and depletion of intracellular potassium, resulting in activation of Ca^{2+}-independent phospholipase A_2 ($iPLA_2$), which induces generation of active caspase-1 that catalyzes the formation of mature IL-1β from proIL-1β (Sluyter et al., 2004). An important function of inflammatory macrophages is phagocytosis and elimination of potentially harmful pathogens. For this purpose, macrophages are equipped with a diverse array of cytotoxic effector functions, including the secretion of proteolytic enzymes and the production of ROS and reactive nitrogen species (RNS) However, uncontrolled cytotoxicity and cell killing may result in considerable collateral damage to healthy tissues. ATP has been shown to modulate macrophage phagocytosis bi-directionally depending on its extracellular concentration. Phagocytosis was inhibited through $P2X_7$ receptor activation at millimolar ATP concentrations, while it was stimulated through activation of

$P2Y_2$ receptors at lower ATP levels (Sung and Silverstein, 1985; Ichinose, 1995). Proteolytic enzyme secretion is affected in a similar bidirectional fashion. Whereas ATP at millimolar levels has been shown to increase lysosomal enzyme secretion via stimulation of $P2X_7$ receptors in macrophages (Labasi et al., 2002), ATP as well as ADP inhibited lysosomal enzyme secretion at micromolar concentrations in one study (Riches et al., 1985).

Dendritic cells

Dendritic cells (DCs) are antigen-presenting innate immune cells whose specific function is to activate naive T cells and to initiate primary immune responses. Human DCs have been shown to express $P2X_{1,4,5,7}$ and $P2Y_{1,2,4,6,11,13,14}$ receptor subtypes (Berchtold et al., 1999; Ferrari et al., 2000; Wilkin et al., 2001; Idzko et al., 2002; Zhang et al., 2002; Schnurr et al., 2003; Skelton et al., 2003; Georgiou et al., 2005). Purinergic receptor expression may depend on degree of DC maturity, since expression of $P2Y_{11}$ and $P2Y_{14}$ receptor subtypes was shown to be downregulated upon maturation of monocyte-derived DCs (MoDCs) (Skelton et al., 2003). ATP at low micromolar concentrations transiently enhanced endocytotic activity of human immature DCs (Schnurr et al., 2000). ATP also appears to affect migration of DCs, as originally reported by Liu and co-workers who demonstrated that DCs migrated towards a pipette containing ATP (Liu et al., 1999). Subsequent studies showed that low micromolar concentrations of ATP as well as UDP and UTP stimulated migration of DCs by activating P2Y receptors (Idzko et al., 2002; la Sala et al., 2002; Schnurr et al., 2003; Idzko et al., 2004b). In contrast, a gradient of ATP and chemokines, which is likely to occur at sites of inflammation or tissue damage in vivo, resulted in inhibition of chemokine-directed migration of human MoDCs through activation of $P2Y_{11}$ receptors (Schnurr et al., 2003). The authors hypothesized that ATP, after initially stimulating DC migration towards sites of inflammation, may cause an arrest in movement of DCs arriving at these inflammatory sites by activating $P2Y_{11}$ receptors, thereby prolonging exposure of DCs to antigens and factors inducing DC maturation. Extracellular ATP may in fact be one of the factors involved in DC maturation, as micromolar ATP concentrations have been shown to induce up-regulation of maturation markers (CD80, CD83, CD86, CD54 and major

histocompatibility complex MHC-II) in human peripheral blood mononuclear cell (PBMC)-derived immature DCs (Schnurr et al., 2000; la Sala et al., 2001;Wilkin et al., 2001, 2002; Marteau et al., 2004), possibly by stimulation of the $P2Y_{11}$ receptor subtype. Purinergic signaling by extracellular nucleotides is thought to modulate cytokine production by DCs, resulting in a specific cytokine profile that appears to depend on which P2 receptor subtype is being activated. The first indication of such an effect came from an experiment showing that murine fetal skin-derived DCs (FSDCs) selected for low $P2X_7$ receptor expression released less IL-1β upon co-incubation with Th lymphocytes than wild-type FSDCs, which express the $P2X_7$ receptor subtype at a high level (Mutini et al., 1999). When coincubated with syngeneic Th cells, the ATP-resistant FSDCs also showed reduced T cell-stimulatory activity in comparison with wild-type cells, as judged by the ability to trigger IL-2 secretion by T cells (Mutini et al., 1999). Prolonged exposure (>15–30 min) to millimolar ATP concentrations causes morphological changes and eventually apoptosis of mouse and human DCs (Mizumoto et al., 2002; Sluyter and Wiley, 2002). These cytotoxic effects of ATP, which were shown to be mediated by the $P2X_7$ receptor and partly depended on activation of caspase-1 and caspase-3 (Coutinho-Silva et al., 1999; Falzoni et al., 2000), may contribute to the removal of DCs after antigen delivery to lymphocytes in lymphoid tissues.

Lymphocytes

Lymphocytes are central to humoral and cellular immune responses, two major lymphocyte subclasses are B and T cells expressing antigen-specific receptors, which are the hallmark of adaptive immunity. T lymphocyte function may be subject to autocrine or paracrine regulation by extracellular nucleotides as suggested by three lines of evidence. First, T lymphocytes are capable of releasing ATP upon activation (Filippini and Sitkovsky, 1990; Canaday et al., 2002; Into et al., 2002b; Loomis et al., 2003). Second, T lymphocytes express purinergic receptors for extracellular nucleotides. Several P2X receptor subtypes are expressed in peripheral T lymphocytes, such as, $P2X_{1,4,5,7}$ (Chused et al., 1996; Gu et al., 2000; Smith et al., 2001; Di Virgilio et al., 2001; Li et al., 2001; Adinolfi et al., 2002; Budagian et al., 2003; Loomis et al., 2003; Wang et al., 2004). Although mRNA of $P2Y_{1,2,4,6,11,12,13,14}$ receptor subtypes has been detected in resting

peripheral T lymphocytes (Jin et al., 1998; Somers et al., 1998; Moore et al., 2001a; Duhant et al., 2002; Zhang et al., 2002; Moore et al., 2003a; Wang et al., 2004), P2Y receptors are thought to be nonfunctional in these cells (Oshimi et al., 1999; Duhant et al., 2002). However, T cell activation during immune responses may induce expression of functional P2Y receptor subtypes. The third line of evidence, which would suggest extracellular nucleotide-mediated regulation of T lymphocyte function, comprises the expression of several purine-metabolizing ecto-enzymes by T lymphocytes. Lymphocytes are thought to be characterized by an ATP regenerating/Adenosine-eliminating phenotype allowing them to maintain surrounding ATP at steady-state levels (Yegutkin et al., 2002). Extracellular ATP has been shown to induce shedding of L-selectin from T lymphocytes via activation of $P2X_7$ receptors (Elliott and Higgins, 2004). Since L-selectin is primarily involved in lymphocyte homing to lymphoid tissues and is shed upon lymphocyte activation, ATP may be involved in migration of activated lymphocytes to sites of inflammation. Purine nucleotides may also indirectly facilitate transendothelial lymphocyte extravasation by impairing vascular barrier function. Henttinen and co-workers demonstrated that adhesion of lymphocytes to endothelial cells suppressed endothelial purine metabolism (Henttinen et al., 2003). Extracellular ATP may be involved in lymphocyte proliferative responses. In murine peripheral T lymphocytes, ATP at concentrations of 0.5–2 mM was shown to inhibit proliferation of both resting and stimulated cells (Ikehara et al., 2004; DosReis et al., 1986). These inhibitory effects might be due to breakdown of ATP to adenosine. Using human peripheral blood lymphocytes, ATP at micromolar levels (100–300 μM) was shown to be a co-stimulator of mitogen-induced CD^{4+} and CD^{8+} T lymphocyte proliferation via activation of a P2X receptor channel, possibly the $P2X_7$ receptor (Baricordi et al., 1996). The $P2X_7$ receptor is expressed on normal B lymphocytes (Collo et al., 1997; Markwardt et al., 1997; Smith et al., 2001), but also on CD^{5+} B lymphocytes from chronic lymphocytic leukemia (CLL) patients (Barden et al., 2003). NK cells are a subset of lymphocytes that appear to have a specific capacity for spontaneous cytolysis of tumor and virus-infected cells. Research on the effects of nucleotides on the activity of NK cells is very limited; existing knowledge derives mainly from studies done in the early 1990s. A study reported a dose-dependent inhibition of NK cell-mediated cytotoxic activity by exogenous ATP, probably by interacting

with P2 purinergic receptors. It was shown that ATP (micromolar) treatment of these NK cells neither induced a non-specific decrease in the number of viable cells, nor showed specific elimination of the NK cells (Krishnaraj, 1992). In human NK cells, incubation with extracellular purine nucleotides resulted in an inhibition of the IL-2 dependent NK proliferation, which was stronger for ATP than for adenosine. Koziak and co-workers showed in human NK cells a correlation between the levels of CD39 expression and ATP diphosphohydrolase (ATPDase) activity (Koziak et al., 1999) The presence of mRNAs specific for purinergic P2 receptors ($P2Y_1$, $P2X_4$, and $P2X_7$) in these NK cells would suggest a regulation of the purinergic signaling by the CD39/ATPDase co-expression.

ATP and adenosine as endogenous signaling molecules in immunity and inflammation

The extensive available data on the immunologic effects of purinergic signaling by extracellular ATP and adenosine, as presented in the previous paragraphs, gives overwhelming evidence that these endogenous signaling molecules and their purinergic receptors play a major role in immunity and inflammation. However, the presented data also suggest that the role of ATP and adenosine in immunity and inflammation is extremely complex and interdependent. Existing knowledge derives mainly from in vitro studies, which are directed towards unravelling biochemical mechanisms at molecular receptor and post-receptor levels, rather than providing a general picture of physiological or pathological conditions in vivo. The immunological role of ATP and adenosine is best viewed within the scope of a model which provides a functional explanation for the initiation of inflammatory and immune responses as well as their resolution, in short, for the regulation of immunity. One theory, which has gained considerable empirical support over the past decade and that complements the classical infectious-nonself theory of immunity, is the so-called “Danger theory”, which proposes that the immune system is principally occupied with detecting “danger”, defined as anything causing tissue damage or cellular stress (Matzinger, 1994, 2001, 2002; Heath and Carbone, 2003; Jerome and Corey, 2004). According to this line of thought, substances causing damage or emerging during, after or because of damage constitute danger signals that alert and instruct the immune system

(Gallucci & Matzinger, 2001; Rock et al., 2004; Skoberne et al., 2004). A distinction is made between on the one hand exogenous danger signals, which are typically the pathogen-associated molecular patterns (PAMPs; e.g. LPS) triggering responses through ligation of pattern-recognition receptors (PRRs; e.g. TLRs), and on the other hand endogenous danger signals, which can be produced by activated immune cells (e.g. cytokines) or can derive from stressed or damaged tissue cells. These latter tissue-derived distress signals have recently also been called damage-associated molecular patterns (DAMPs) (Seong and Matzinger, 2004). DAMPs initiate and regulate immune responses in co-operation with other danger signals, and should ideally be entities that: (1) are constitutively present at high intracellular concentrations, (2) are normally present at negligible extracellular concentrations, (3) are easily released in response to injury, infection or other inflammatory stimuli, (4) are able to activate selective and specific cellular receptors responsive over a wide range of concentrations, and (5) are quickly degraded following their release (Di Virgilio et al., 2003; Di Virgilio, 2005). Both ATP and adenosine meet all five of the above prerequisites, and may therefore be considered as DAMP molecules. For the purpose of clarity, inflammatory and immune responses are divided into three stages, which partly overlap (Chaplin, 2003; Gilroy et al., 2004; Sherwood and Toliver-Kinsky, 2004). The first stage encompasses the onset of acute inflammation and the initiation of primary immune responses upon encounter with infectious or injurious agents. The second stage comprises the modulation and fine-tuning of ongoing inflammatory and immune responses by endogenous immunoregulatory substances. Finally, the third stage encompasses the down-regulation of immune responses, the induction of inflammatory resolution and the restoration of damaged tissues to preserve cellular homeostasis. These three stages will be used as a steppingstone to positioning purinergic signaling by extracellular ATP and adenosine in immunity and inflammation. In the first stage, extracellular ATP mainly functions as a proinflammatory and immunostimulatory mediator in the microenvironment of damaged cells. ATP may be part of a group of endogenous molecules that have recently been termed ‘alarmins’ (Oppenheim and Yang, 2005). These multifunctional molecules appear to be an unique subgroup of endogenous danger signals since they exhibit both chemotactic and activating effects on leukocytes, particularly DCs, thereby displaying potent innate immuno-

enhancing activity (Oppenheim and Yang, 2005). ATP is present in the cell cytosplasm at millimolar concentrations and is released from the intracellular compartment upon cellular stress or non-physiological necrotic cell death. Extracellular ATP concentrations in the local microenvironment of damaged cells can rise considerably, marking the damaged site and contributing to the promotion of inflammation and the initiation of primary immune responses. From this point of view, ATP at high extracellular concentrations appears to be a natural endogenous adjuvant released from injured and dying cells, which initiates inflammation and has an augmenting effect to amplify and sustain cell-mediated immunity through P2 receptor-mediated purinergic signaling. At an early phase following cell damage, when ATP levels are highest, the receptor subtype most likely involved in sensing the purinergic danger is the $P2X_7$ receptor (Elliott and Higgins, 2004). Via activation of $P2X_7$ receptors, ATP induces the production of cytokines such as IL-1β, IL-2, IL-12, IL-18 and TNFα by residing immune cells, triggering the inflammatory response and inducing type 1 lymphocyte polarization. Highlevel signaling through other P2 receptors probably also contributes to the pro-inflammatory and immunostimulatory role of ATP. Recruitment of leukocytes to damaged sites is promoted by ATP as well as other adenine and uridine nucleotides, which also induce inflammatory activation of neutrophils, classical activation of macrophages, maturation and Th1 cell-stimulatory capacity of DCs, and proliferation and activation of lymphocytes. In the second stage, the immunological role of ATP appears to shift gradually from being mostly immunostimulatory to being more immunomodulatory. In addition, extracellular adenosine mainly appears to predominate as an anti-inflammatory mediator at this stage. Over-activation of the immune system may lead to uncontrolled or chronic inflammation resulting in collateral cell damage and destruction of healthy tissues. Therefore, inflammatory and immune responses must be tightly regulated to protect the host. For this purpose, the immune system disposes of several regulatory molecules that orchestrate host responses by controlling inflammatory and immune responses (Nathan, 2002). Extracellular ATP and adenosine could be endogenous regulatory molecules comprising a purinergic feedback system. Crucial components of such a purinergic feedback system are the purinergic receptors mediating the immunological effects of extracellular nucleotides and nucleosides, as well as the ectoenzymes mediating a

purinergic cascade that leads to a progressive decrease in nucleotide concentrations and an increase in nucleoside concentrations. The expression profile of purinergic receptors as well as of ecto-enzymes by immune cells changes under inflammatory conditions, allowing for the progressive acquisition of an immunomodulatory purinergic repertoire expressed by the cells involved in inflammatory and immune responses. The ecto-enzymes CD39 and CD73 control extracellular nucleotide concentrations and thereby regulate the extent of purinergic signaling, as evidenced by several studies using ecto-enzyme knock-out models (Goepfert et al., 2000, 2001; Mizumoto et al., 2002; Eltzschig et al., 2003, 2004; Koszalka et al., 2004). It appears that low-level purinergic signaling by P2 receptors, induced by nucleotides at decreased concentrations, modulates ongoing inflammatory and immune responses. Activation of P2 receptors, most probably P2Y receptor subtypes, attenuates pro-inflammatory cytokine production by monocytes and macrophages, diminishes Th1 cell-stimulatory capacity of DCs and inhibits lymphocyte effector functions. Thus, upon progression of the immune response, nucleotide-mediated purinergic signaling may switch from being predominantly pro-inflammatory to being mostly immunomodulatory, depending on the extracellular concentrations of the nucleotides as well as the P2 receptor subtype(s) ligated by these nucleotides. This switch could be part of a mechanism by which the effector class of an immune response is being fine-tuned and re-directed by microenvironmental signals according to the tissue in which the response occurs (Di Virgilio et al., 2003; La Sala et al., 2003; Mazzoni and Segal, 2004). However, evidence for such an immunomodulatory role of low-level ATP-mediated purinergic signaling is very limited. ATP at low micromolar concentrations markedly inhibited the inflammatory reaction by inhibiting TNFα release and stimulating IL-10 release in immunostimulated whole blood, even under conditions of severe oxidative stress (Swennen et al., 2005, 2006). Since natural cell-to-cell interactions are preserved in this diluted whole blood system and blood components are present in in vivo ratios with non-cellular components, this ex vivo system is a good reflection of the natural environment. Furthermore, adenosine-mediated P1 receptor signaling during the second stage down-regulates neutrophil effector functions, contributes to alternative activation of macrophages, stimulates Th2 cell-stimulatory capacity of DCs and inhibits lymphocyte effector functions. At the final stage, extracellular adenosine appears to be an important

immunosuppressive and tissue-healing factor. Like ATP, adenosine can also be considered as a danger molecule because its extracellular levels rise markedly in response to tissue damage. However, unlike ATP, rising extracellular adenosine levels in response to damage inflicted by overactive host immune cells mediate an autoregulatory immunosuppressive loop to protect healthy tissues from these 'dangerous' immune cells. Damage-associated increases in extracellular adenosine thus represent a 'second' danger signal that is sensed by P1 receptors (Frantz et al., 2005). Inflammatory mediators progressively up-regulate the expression of the P1 receptor subtypes through which adenosine mediates its immunosuppressive effects (Varani et al., 2003; Gessi et al., 2004b; Lappas et al., 2005a; Murphree et al., 2005; Fortin et al., 2006). Using receptor knock-out models, the A_{2A} AR subtype has been considered to play a non-redundant role in down-regulating cell-mediated immunity and in activating pro-resolution pathways (Thiel et al., 2005; McColl et al., 2006). A_1, A_{2B} and A_3 ARs also contribute to the adenosine-mediated negative feedback signaling (Lee et al., 2004a, 2004b; Tsutsui et al., 2004; Sun et al., 2005; Tilley and Boucher, 2005). Adenosine mediated signaling by these purinergic receptors induces an angiogenic switch in macrophages or deactivates macrophages, suppresses proliferation and effector functions of lymphocytes, and promotes angiogenesis and tissue regeneration. In conclusion, although it is still far from clear how extracellular ATP and adenosine exert their immunologic roles, they appear to be crucial endogenous signaling molecules in immunity and inflammation. Because ATP and adenosine are unstable molecules with a short half-life, they probably operate only transiently in the local microenvironment of cells in an autocrine or paracrine manner. Purinergic signaling constitutes one of the mechanisms by which the immune system tailors inflammatory and immune responses according to the host's need for protection against danger. Purinergic signaling molecules may be beneficial in the treatment of immune-related diseases, as signaling by extracellular adenosine may contribute to the anti-inflammatory effects of low-dose methotrexate, which probably is one of the most prescribed anti-inflammatory drug in the treatment of patients with RA (Cronstein, 2005). Thus in view of the pivotal role of the immune system in health and disease, current research on endogenous immunomodulatory molecules, ATP and adenosine included, may help devise novel strategies for the treatment of human diseases.

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AIM OF THE THESIS

The nucleotide ATP is normally present in every living cell of the human body and is well-known for its role in intracellular energy metabolism. In addition to this intracellular role, ATP in the extracellular compartment is thought to contribute to the regulation of a variety of other biological processes, including cardiac function, neurotransmission, muscle contraction, vasodilatation, bone metabolism, liver glycogen metabolism and inflammation. The human immune system comprises an interactive network of lymphoid organs and immune cells, and is essential to host defense. Interaction between the various components of the immune system during activation is realized by multiple signaling molecules. These molecules, which can be released in response to tissue injury or exogenous pathogens, signal danger to the host and are necessary for initiating primary immune responses as well as for controlling the course and resolution of the concomitant inflammatory processes. Extracellular nucleotides such as ATP may function as endogenous signaling molecules that control inflammation and immune responses. Modulation of inflammatory processes by extracellular ATP is complex and results from specific effects on a wide variety of both immune and non-immune cells. The role of ATP in immunity is closely related to one of its breakdown products, the nucleoside adenosine. Adenosine has an already established role in immunity, in which it may contribute to the engineering of inflammation and immune reponses by providing a suppressive tissue-protecting signal in a delayed, negative feedback manner. Animal models of asthma, ischaemia, arthritis, sepsis, inflammatory bowel disease and wound healing have helped to elucidate the regulatory roles of the various adenosine receptors in dictating the development and progression of disease. The notion of an interrelation between ATP and adenosine is firmly based on the presence of a large family of purinergic receptors. Several enzymes, which are also expressed by various immune and non-immune cells, are involved in a purinergic cascade by which extracellular purine levels and the ensuing purinergic signaling can be dynamically controlled during inflammatory. This recent heightened awareness of the role of ATP and adenosine in the control of immune and inflammatory systems has generated excitement regarding the potential use of purinergic-based therapies in the treatment of inflammatory diseases.

On this background, the aim of this study is to investigate the role of A_1, A_{2A}, A_{2B} and A_3 ARs, $P2X_1$ and $P2X_3$ in chondrocytes and fibroblast-like synoviocytes that are the cells mainly involved in inflammatory processes of joint diseases such as osteoarthritis and rheumatoid arthritis. The study in **chapter 1** describes the presence and binding parameters of A_1, A_{2A}, A_{2B} and A_3 ARs in bovine chondrocytes and fibroblast-like synoviocytes. To this aim saturation, competition binding experiments and western blotting assays to adenosine receptors in bovine chondrocytes and fibroblast-like synoviocytes were performed. In the adenylyl cyclase and proliferation assays the potency of typical high-affinity agonists was evaluated to characterized the functional responses of ARs. In the **chapter 2** the role of adenosine analogs stimulation on prostaglandin E_2 (PGE_2) release and cyclooxygenase-2 (COX-2) expression in bovine fibroblast-like synoviocytes was investigated. Saturation and competition binding experiments were performed by using adenosine agonists such as CHA, CGS 21680, NECA and Cl-IB-MECA. Bovine fibroblast-like synoviocytes were treated with TNF-α and lipopolysaccharide (LPS) to activate inflammatory response. Adenosine analogs were added to control and TNF-α- or LPS- treated cultures both in the absence and presence of adenosine deaminase (ADA) which is used to depleted endogenous adenosine. The modulation of PGE_2 release was measured by immunoassay and COX-2 expression was evaluated by RT-PCR. **Chapter 3** reports the pharmacological and functional characterization of ARs in human fibroblast-like synoviocytes derived from patients with OA. To this aim mRNA, western blotting, saturation binding experiments and cAMP assays were performed. To better investigate the functional role of adenosine in human fibroblast-like synoviocytes, the activation of MAPK and NF-κB and the release of TNF-α and IL-8 were analized. In **chapter 4** thermodynamic parameters such as standard free energy, enthalpy and entropy ($\Delta G°$, $\Delta H°$ and $\Delta S°$ respectively) of the binding equilibrium of well-known purinergic agonists and antagonists to human $P2X_1$ and $P2X_3$ receptors, expressed in HEK-293 cells, were determined. To this aim saturation binding experiments were performed by using [^{3}H]αβmethyleneATP as radioligand, and the van't Hoff analysis were conducted with the aim to investigate the driving forces associated with the drug-receptor interaction. The thermodynamic characterization of human purinergic $P2X_1$ and $P2X_3$ expressed in HEK-293 cells, represents an important point to better

investigate purinergic receptors in native tissues. As a consequence, in **chapter 5** a biochemical and functional characterization of $P2X_1$ and $P2X_3$ receptors were performed in bovine chondrocytes. In particular saturation, competition binding experiments, western blotting, thermodynamic analysis and immunohistochemistry assays in bovine chondrocytes were performed to better analized purinergic receptors. The pharmacological characterization of purine receptors was completed by functional assays to evaluated the effect of purinergic agonists and antagonists on NO and PGE_2 release. The study in **chapter 6** describes, in fibroblast-like synoviocytes obtained from OA patients and in SW 982 cells derived from human synovial sarcoma, a pharmacological characterization of $P2X_1$ and $P2X_3$ receptors. To this aim mRNA, western blotting, saturation and competition binding experiments were performed to characterize the purinergic receptors. In addition NF-κB activation, TNF-α, IL-6 and PGE_2 production were also evaluated by means of enzyme-linked immunosorbent assays.

CHAPTER 1:

Characterization of adenosine receptors in bovine chondrocytes and fibroblast-like synoviocytes.

INTRODUCTION

Chronic inflammation is a significant factor in the pathophysiology of many forms of joint disease. Adenosine has been reported to reduce inflammation in several in vivo models suggesting a potential value of this purine nucleoside as a therapeutic mediator of inflammatory joint disease able to limit articular cartilage degeneration (Tesh et al., 2002, 2004). In the peripheral system, adenosine has been shown to limit systemic inflammatory responses through receptor-mediated regulation of a wide variety of cell types (Varani et al., 1997; Gessi et al., 2004). Adenosine interacts with four cell surface receptor subtypes named as A_1, A_{2A}, A_{2B} and A_3, which are coupled to different G-proteins (Fredholm, 2001). A_1 and A_3 receptors mediate inhibition of the adenylate cyclase activity, in contrast both A_{2A} and A_{2B} subtypes stimulate cAMP (cyclic AMP) accumulation. A role of adenosine in modulating chondrocytes and fibroblast-like synoviocytes is documented by previous studies. In chondrocytes it has been reported that adenosine and its analogs were capable of altering cAMP and nitric oxide (NO) production (Tesh et al., 2002, 2004). In addition, it seems likely that for normal cartilage function, extracellular adenosine levels must be quite tightly regulated since depletion leads to increased glycosaminoglycan (GAG) release and the production of matrix metalloproteinases (MMPs) such as MMP-3 and MMP-13, prostaglandin E_2 (PGE_2) and NO, whilst its increase may trigger chondrocyte death (Tesh et al., 2004; Benton et al., 2002; Mistry et al., 2006). In fibroblast-like synoviocytes adenosine receptor stimulation is involved in the regulation of MMPs (Boyle et al., 1996, 1997). Recently, in human synoviocytes it has been reported the selective involvement of the A_{2A} AR subtype in the immunomodulatory actions of methotrexate (Ralph et al., 2005) Further, in vivo adenosine A_{2A} AR agonists inhibit cartilage damage when used in the treatment of septic arthritis by diminishing interleukin-8 expression and GAG loss and reduce rat adjuvant induced arthritis (Boyle et al., 2002; Cohen et al., 2004, 2005). Although A_{2A} and A_{2B} ARs transcripts have been described in articular chondrocytes (Koolpe et al., 1999), however, the binding parameters such as affinity and density of adenosine subtypes have not been investigated in chondrocytes and fibroblast-like synoviocytes. From this background, the aim of this study was to investigate the expression and binding parameters of the A_1, A_{2A},

A_{2B} and A_3 ARs in bovine chondrocyte and fibroblast-like synoviocyte membranes. The ARs most involved in inflammatory process were the subtypes A_{2A} and A_3 thus we also performed competition experiments, cAMP levels and cell proliferation assays of A_{2A} or A_3 ARs agonists.

MATERIALS AND METHODS

Chondrocyte monolayer cultures

Bovine articular cartilage derived from the metacarpophalangeal joints of 14-18-month-old animals (Limousine breed). Chondrocytes were isolated from cartilage fragments obtained from the weight-bearing region of the articular surface (Pezzetti et al., 1999). Briefly, the cartilage was dissected out and cut into small pieces. Pieces were subjected to a sequential digestion in Dulbecco's modified Eagle's/Ham's F12 (1:1) medium (DMEM/F12) with pronase from Streptomyces griseus for 90 min and collagenase P from Clostridium histolyticum for about 12 h. The resulting cell suspension was filtered to remove undigested cartilage. Chondrocytes were recovered by centrifugation, counted and plated at high density (150,000/cm^2) in 75 cm^2 flasks and in multiwells (6.6 x 6.6 cm, 1.6 cm the diameter of each well). Chondrocytes were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 U/ml, streptomycin 0.1 mg/ml) (complete medium). Only chondrocytes without subculturing and maintained in culture for 1 week were used in the binding and functional experiments.

Fibroblast-like synoviocyte cultures

Fibroblast-like synoviocytes were obtained by culture of the bovine synovial fluid. Synovial fluid was aspirated from the metacarpophalangeal joints of 14-18-month-old animals by a syringe. Then, fresh synovial fluid was diluted 1:4 with complete medium and plated in 25 cm^2 culture flasks (Falcon, Becton Dikinson and Company, Franklin Lakes, NJ, USA). After 3 h, medium was removed and fresh complete medium was added to the flasks. Cells were maintained in culture and passaged when reaching confluence. Synovial cells at the third and fourth passages were used for the analysis of adenosine receptors.

Chondrocytes and fibroblast-like synoviocytes characterization

The expression of aggrecan, type II and type I collagens in the same chondrocyte monolayer cultures used for binding experiments was evaluated by immunohistochemistry as previously described (De Mattei et al., 2004). Incubations with the primary mouse monoclonal antibody to aggrecan (1:50) and the rabbit polyclonal to collagen type II (1:200) (Abcam, Cambridge Science Park, Cambridge, UK) were performed for 1 h at 37°C. Fibroblast-like synoviocytes were characterized by immunofluorescence staining with vimentin, a specific cellular marker for mesenchymal cells (Upragarin et al., 2005). Cells were fixed with cold methanol, washed with phosphate buffer saline (PBS) and incubated with the primary mouse antibody specific for the human vimentin at 1:200 dilution for 1 h at 37°C. Washed slides were then incubated with a secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibody for 1 h at 37°C in the dark. Antibodies were from Sigma-Aldrich S.r.l. (Milan, Italy). Fluorescence was visualized using the Nikon Eclipse TE 2000-E microscope (Nikon Instruments Spa, Sesto Fiorentino, Firenze, Italy) equipped with a digital camera (DXM 1200F, Nikon Instruments Spa, Sesto Fiorentino, Firenze, Italy). To confirm that fibroblast-like synoviocyte cultures were not contaminated by macrophages, CD14 expression was evaluated by reverse transcription polymerase chain reaction (RT-PCR). Total RNA extraction was performed using a commercial kit. RNA conversion to cDNA was performed using the commercial kit Superscript First-strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA, USA). Two microliters of cDNA were amplified by specific oligonucleotide primers for CD 14 (dp50-CTG GAA GCC GGC G-30; rp50-AGC TGA GCA GGA ACC TGT GC-30). Primer sequences were selected to amplify both human and bovine genome and were from separate exons to exclude a possibly genomic DNA contamination of the RNA samples. PCR reactions were performed in a total volume of 25 ml containing 1 U Taq DNA polymerase (Roche Molecular Biochemicals, Indiana, USA), 25 pmol of each primer, 200 mM deoxynucleotide triphosphates (dNTPs) in 1 x PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$). Cycling parameters were as follows: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C for CD14. The size of the amplified sequence was 403 bp. mRNA from human macrophages was used as a

positive control for CD14 expression. PCR products were analyzed on 1.5% agarose gel, stained with ethidium bromide.

Preparation of bovine chondrocytes and fibroblast-like synoviocytes membranes

For membrane preparation, the culture medium was removed the cells were washed with PBS and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA pH 7.4). The cell suspension was homogenized by using a Polytron and was centrifuged for 30 min at 100,000g. The membrane pellet was resuspended in the same buffer solution used in the binding experiments, incubated with 2 IU/ml of adenosine deaminase for 30 min at 37°C and centrifuged for 30 min at 100,000g. Finally the suspension was used in saturation and competition binding experiments. The protein concentration was determined according to a Bio-Rad method with bovine albumin as reference standard.

Western blotting of ARs

Chondrocytes and fibroblast-like synoviocytes 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, and 1.4 mM E-64. Then cells were lysed in Triton lysis buffer and the protein concentration was determined using bicinchoninic acid (BCA) protein assay kit (Pierce, Illinois, USA). Aliquots of total protein sample (50 mg) were analyzed using antibodies specific for human A_1, A_{2A}, A_{2B} and A_3 ARs (1 mg/ml dilution) (Merighi et al., 2002). Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies (1:2000 dilution). Specific reactions were revealed with Enhanced Chemiluminescence Western blotting detection reagent (Amersham Biosciences, New York, USA).

Saturation and competition binding experiments to ARs

The chondrocyte and synoviocyte membranes were used for the A_1, A_{2A}, A_{2B} and A_3 binding experiments (Varani et al., 2002, 2003a). Saturation binding experiments to A_1 ARs were performed according to the method described previously using [^{3}H]-1,3-dipropyl-8-cyclopentyl-xanthine ([^{3}H]-DPCPX, specific activity 120 Ci/mmol; NEN-Perkin Elmer Life and Analytical Sciences, USA) as

radioligand (Borea et al., 1994). The membranes derived from chondrocytes or fibroblast-like synoviocytes (100 mg of protein/assay) with 8-10 concentrations of the radioligand [^{3}H]-DPCPX (0.01-20 nM) were incubated in Tris HCl 50 mM, pH 7.4, for 90 min at 4°C. Non specific binding was determined in the presence of DPCPX 1 μM. Saturation binding experiments to A_{2A} ARs were performed according to the method described previously using [^{3}H]-4-(2-[7-amino-2-(2-furyl)[1,2,4] triazolo [2,3-a] [1,3,5] triazin-5-yl-amino]-ethyl ([^{3}H]-ZM 241385, specific activity 27.4 Ci/mmol; American Radiolabeled Chemicals Inc, Saint Louis, MO, USA) as radioligand (Varani et al., 2003b). The membranes derived from chondrocytes or fibroblast-like synoviocytes (100 mg of protein/assay) were incubated for 60 min at 4°C with 8-10 concentrations of the radioligand [^{3}H]-ZM 241385 (0.01-20 nM) and Tris HCl 50 mM, $MgCl_2$ 10 mM, pH 7.4. Nonspecific binding was determined in the presence of ZM241385 1 μM. Saturation binding experiments to A_{2B} ARs were performed using [^{3}H]-N-benzo [1,3[dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]-acetamide ([^{3}H]-MRE 2029F20, specific activity 123 Ci/mmol; Amersham International Chemical Laboratories, Buckinghamshire, UK) as radioligand (Gessi et al., 2005). The membranes obtained as previously described (100 mg of protein/assay) with 8-10 concentrations of [^{3}H]-MRE 2029F20 in the range 0.01-20 nM were incubated in Tris HCl 50 mM, $MgCl_2$ 10 mM, EDTA 1 mM, pH 7.4 at 4°C for 60 min. Nonspecific binding was determined in the presence of MRE 2029F20 1 μM. Saturation binding experiments to A_3 ARs were performed using [^{3}H]-5N-(4-methoxyphenylcarbamoyl) amino-8-propyl-2-(2-furyl) pyrazolo [4,3-e]-1,2,4-triazolo [1,5-c]pyrimidine ([^{3}H]-MRE 3008F20, specific activity 67 Ci/mmol; Amersham International Chemical Laboratories, Buckinghamshire, UK) as radioligand (Varani et al., 2000). The membranes treated as above mentioned (100 mg of protein/assay) with 8-10 concentrations in the range 0.01-50 nM of [^{3}H]-MRE 3008F20 were incubated in Tris HCl 50 mM, $MgCl_2$ 10 mM, EDTA 1 mM, pH 7.4, at 4°C for 150 min. Nonspecific binding was determined in the presence of MRE 3008F20 1 μM. In competition experiments, carried out to determine the A_{2A} or A_3 affinity values, 1 nM of [^{3}H]-ZM241385 or 2 nM of [^{3}H]-MRE3008F20, bovine chondrocyte or fibroblast-like synoviocyte membranes (100 mg of protein per assay) and at least 6-8 different concentrations of 2-[p-(2-carboxyethyl)-phenetyl-amino]-50-N-ethyl-

carboxamido adenosine (CGS 21680, Sigma-RBI, St. Louis,MO,USA) or N6-(3-iodobenzyl)2-chloroadenosine-50-N-methyluronamide (Cl-IB-MECA, Sigma-RBI, St. Louis, MO, USA) as typical A_{2A} or A_3 adenosine agonists were incubated at 4°C for 60 or 150 min, respectively. In saturation or competition binding experiments, at the end of the incubation time, bound and free radioactivity was separated by filtering the assay mixture through Whatman GF/B glass fiber filters by using a Brandel cell harvester. The filter bound radioactivity was counted by Scintillation Counter Packard Tri Carb 2500 TR with an efficiency of 58%.

Measurement of cAMP levels in bovine chondrocytes or fibroblast-like synoviocytes

Bovine chondrocytes or fibroblast-like synoviocytes (10^6 cells/ml) were suspended in 0.5 ml incubation mixture Krebs Ringer phosphate buffer, containing 1.0 IU/ml adenosine deaminase and 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor and preincubated for 10 min in a shaking bath at 37°C. Then the effect of a typical A_{2A} adenosine agonist was studied by using CGS 21680 at different concentrations (1 nM-1 μM) that was added to the mixture for a further 5 min. In similar experimental conditions, the effect of N-ethylcarboxamidoadenosine (NECA) on adenosine nonselective agonist was studied. To evaluate the effect of a typical A_3 adenosine agonist, forskolin 1 μM and Cl-IB-MECA at different concentrations (0.1 nM-100 nM) were added to the mixture and the incubation continued for a further 5 min. The effect of a selective A_{2A} or A_3 antagonists such as 7-(2-phenylethyl)-2-furyl)pyrazolo [4,3-e]-1,2,4-triazolo-[1,5-c] pyrimidine (SCH 58261) (1 mM) or MRE 3008F20 (1 mM) on CGS 21680(1 mM) or Cl-IB-MECA (100 nM) was evaluated, respectively. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The cells were also incubated with forskolin (1 mM) and/or Ro 20-1724 (0.5 mM) to evaluate the adenylyl cyclase activity. The TCA suspension was centrifuged at 2000g for 10 min at 4°C and the supernatant was extracted four times with water saturated diethyl ether. The final aqueous solution was tested for cAMP levels through a competition protein binding assay by using [^{3}H]-cAMP as radioligand (specific activity 21 Ci/mmol, NEN Research Products, Boston, MA, USA) (Varani et al., 2002). Samples of cAMP standards (0-10 pmol) were added to each test tube containing

trizma base 0.1 M, aminophylline 8.0 mM, mercaptoethanol 6.0 mM, pH 7.4 and [^{3}H]-cAMP (at the final concentration of 1 nM). The binding protein, previously prepared from beef adrenals, was added to the samples and incubated at 4°C for 150 min. At the end of the incubation time and after the addition of charcoal the samples were centrifuged at 2000g for 10 min. The clear supernatant was mixed with 4 ml of Atomlight and counted in a Scintillation Counter Packard Tri Carb 2500 TR.

[^{3}H] Thymidine incorporation

Chondrocytes or fibroblast-like synoviocytes were treated with CGS 21680 and Cl-IB-MECA (10 μM) in complete medium containing adenosine deaminase (ADA) and 1 mCi/ml [^{3}H] thymidine. Cells cultured in the absence of adenosine agonists were used as controls. After 24 h of labeling cells were trypsinized, and [^{3}H] thymidine was evaluated as previously described (Merighi et al., 2002). In all cultures, cell viability was evaluated by the Trypan blue exclusion test (Merighi et al., 2005).

Statistical analysis

A weighted nonlinear least-squares curve fitting program Ligand (Munson and Rodbard, 1980) was used for computer analysis of saturation and competition binding experiments. Functional experiments were calculated by nonlinear regression analysis using the equation for a sigmoid concentration-response curve (GraphPAD Prism, San Diego, CA, USA). Analysis of data was done with Student's t test (unpaired analysis). Differences were considered significant at a value of $p<0.01$. All data are reported as mean ± S.E.M. of independent experiments (n=3-6).

RESULTS

Phenotype characterization of bovine chondrocytes and fibroblast-like synoviocytes

Chondrocytes, cultured in monolayer, show phenotypic instability and can dedifferentiate toward the fibroblast phenotype shifting from the synthesis of type II to type I collagen (De Mattei et al., 2004). When analyzed by

immunohistochemistry, under the same experimental conditions of binding experiments, chondrocytes stained positive for aggrecan and type II collagen, specific markers of the chondrocytic phenotype, and resulted negative for type I collagen, which is expressed in dedifferentiated chondrocytes. Negative controls, obtained without primary antibodies, did not show any staining. Fibroblast-like synoviocytes used in our experiments showed the expression of vimentin, the main intermediate filament protein in mesenchymal cells and synovial fibroblast. Results obtained by RT-PCR showed the absence of CD14, thus indicating the absence of contaminating macrophages in our cultures (data not shown).

Western blotting

Figure 1.1A shows the immunoblot signals of A_1, A_{2A} and A_{2B} ARs in bovine chondrocytes and fibroblast-like synoviocytes. The intensity of each band in immunoblot assay was quantified using molecular analyst/PC densitometry software (Bio-Rad). Mean densitometry data from three independent experiments were normalized to control that was set to 100% (figure1.1B-C). Unfortunately Western blot of A_3 AR was undetectable probably due to the low degree of homology between bovines and humans.

Saturation binding experiments in bovine chondrocytes and fibroblast-like synoviocytes

A series of experiments were carried out to determine the binding parameters of A_1, A_{2A}, A_{2B} and A_3 ARs in bovine chondrocytes and fibroblast-like synoviocytes. Table 1.1 reports the affinity (K_D, nM) and density (Bmax, fmol/mg protein) of A_1, A_{2A}, A_{2B} and A_3 ARs in bovine chondrocyte and in fibroblast-like synoviocyte membranes. The affinity values were in the low nanomolar range for both the substrates. Bmax values confirm the presence of all the ARs. For chondrocytes membrane the Bmax values were 41, 53, 51,and 79 fmol/mg protein for A_1, A_{2A}, A_{2B} and A_3 ARs respectively. For the fibroblast-like synoviocytes the Bmax values were 30, 76, 84 and 83 fmol/mg protein for A_1, A_{2A}, A_{2B} and A_3 ARs respectively (figure 1.2-1.3).

Competition binding experiments in bovine chondrocytes and fibroblast-like synoviocytes

Figure 1.4 and figure 1.5 shows the competition curves of CGS 21680 and Cl-IB-MECA in bovine chondrocyte or in fibroblast-like synoviocyte membranes, respectively. The affinity (Ki) of CGS 21680 or Cl-IBMECA in bovine chondrocyte membranes was 28 ± 3 or 2.6 ± 0.3 nM, respectively (n = 4). In fibroblast-like synoviocyte membranes CGS 21680 or Cl-IBMECA showed a Ki value of 30 ± 3 or 3.3 ± 0.3 nM, respectively (n = 4). The competition curves of both the agonists examined, exhibited Hill coefficients near the unity and were best described by the existence of one high-affinity binding site.

cAMP assay in bovine chondrocytes and fibroblast-like synoviocytes

The A_{2A} ARs are coupled to stimulation of adenylate cyclase via Gs stimulatory proteins, which leads to an increase of cAMP formation. Bovine chondrocyte or fibroblast-like synovial cells did not reveal change of basal enzyme activity and of the response of adenylate cyclase activator forskolin. No change in cAMP production was also observed in the absence or in the presence of the cAMP-dependent phosphodiesterase inhibitor, Ro 20-1724 (Table 1.2). We have also evaluated the effect of a typical A_{2A} adenosine agonist such as CGS 21680 on the adenylate cyclase activity. When CGS 21680 was incubated with examined cells an amplification of adenylate cyclase response was detected revealing a significant increase of cAMP production in a concentration-dependent manner. In particular CGS 21680 elicited a stimulation of cAMP levels in bovine chondrocytes with an EC_{50} of 82 ± 7 nM (Figure 1.6A). Moreover, CGS 21680 elicited a stimulation of cAMP levels in fibroblastlike synoviocytes with an EC_{50} of 91 ± 8 nM (Figure 1.7A). The selective A_{2A} antagonist SCH 58261 (1 μM) totally inhibited the rise in cAMP levels induced by CGS 21680 (1 μM) suggesting that the stimulatory effect was essentially A_{2A} mediated (Table 1.2). On the contrary, the A_3 ARs are coupled to inhibition of adenylyl cyclase via Gi proteins, which leads to decreases of cAMP formation. We have also evaluated the inhibitory effect of a typical A_3 adenosine agonist like Cl-IBMECA on the adenylyl cyclase activity. Cl-IB-MECA determined a decrease of cAMP levels in bovine chondrocytes with an IC_{50} values of 6.33 ± 0.64 nM (Figure 1.6B). Moreover Cl-IB-MECA induced a decrease of cAMP levels in fibroblast-like

synoviocytes with an IC_{50} values of 7.90 ± 0.71 nM (Figure 1.7B). The selective A_3 antagonist MRE 3008F20 (1 μM) antagonized Cl-IB-MECA (100 nM) mediated cAMP inhibition suggesting that the inhibitory effect was essentially A_3 mediated (Table 1.2). NECA, a typical adenosine receptor agonist, determines a stimulation of cAMP levels in bovine chondrocytes with an EC_{50} value of 40 ± 3 nM. Analogous results were obtained in fibroblast-like synoviocytes with an EC_{50} value of 37 ± 3 nM. This stimulatory effect due to the interaction of NECA with A_{2A} and A_{2B} ARs predominates on the inhibitory effect mediated by A_1 and A_3 ARs.

Cell proliferation and viability

Chondrocytes or fibroblast-like synoviocytes treated with CGS 21680 or Cl-IB-MECA (10 μM) showed no statistically significant modulation of cell proliferation (Figure 1.8). However, in fibroblast- like synoviocytes, Cl-IB-MECA induced a slight reduction of cell proliferation. In all tested conditions cell viability was not changed with respect to control conditions (99% viable cells).

Table 1.1: Saturation binding experiments on ARs in bovine chondrocyte and in fibroblast-like synoviocyte membranes.

	Chondrocyte membranes		**Fibroblast-like synoviocyte membranes**	
	K_D (nM)	Bmax (fmol/mg protein)	K_D (nM)	Bmax (fmol/mg protein)
A_1 AR	2.18±0.22	41±2	0.67±0.01	30±2
A_{2A} AR	1.71±0.32	53±5	2.05±0.17	76±6
A_{2B} AR	2.19±0.24	51±3	1.21±0.13	84±5
A_3 AR	4.61±0.35	79±5	1.86±0.22	83±4

Table 1.2: Basal and stimulated cAMP production in bovine chondrocytes and in fibroblast-like synoviocytes.

	Chondrocytes (pmol cAMP x 10^6 cells)	**Fibroblast-like synoviocytes (pmol cAMP x 10^6 cells)**
Basal levels	10±1	11±1
+ Ro 20-1724 (0.5 mM)	25±2	23±2
+ CGS 21680 (1 μM) A_{2A} stimulation	51±4*	50±4*
+ CGS 21680 (1 μM) + SCH 58261 (1 μM)	27±2	25±2
Forskolin (1 μM)	98±10	96±9
Forskolin (1 μM) Ro 20-1724 (0.5 mM)	120±12	112±11
+ Cl-IBMECA (100 nM) A_3 inhibition	82±6*	84±6*
+ Cl-IBMECA (100 nM) + MRE 3008F20 (1 μM)	116±11	108±11

*, $p<0.01$ vs basal levels + Ro 20-1724 for CGS 21680 and vs Forskolin + Ro 20-1724 for Cl-IB-MECA

Figure 1.1: Western blotting analysis of bovine chondrocytes and fibroblast-like synoviocytes for A_1, A_{2A} and A_{2B} ARs (A) and densitometric analysis for chondrocytes (B) and fibroblast-like synoviocytes (C).

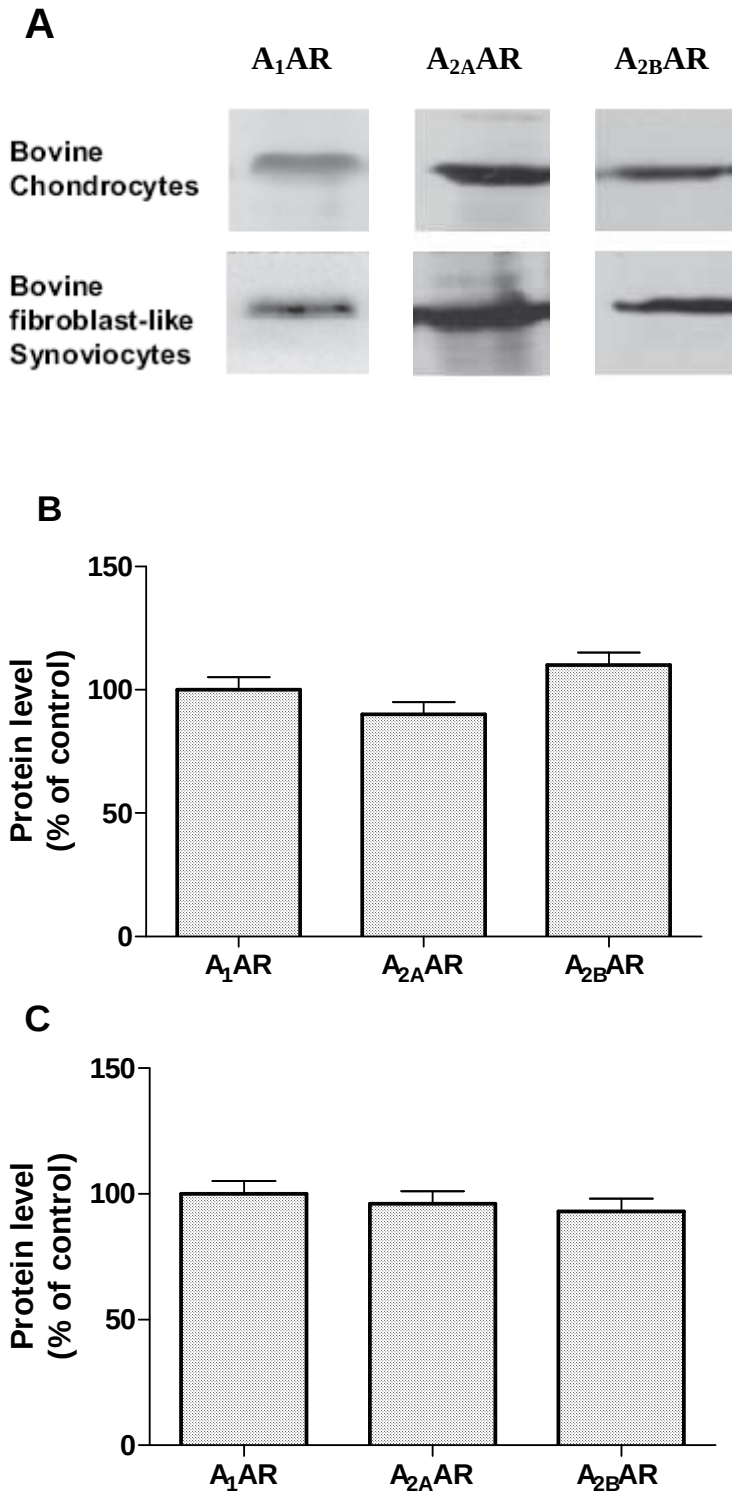


Figure 1.2: Saturation curves and Scatchard plots of A_1, A_{2A}, A_{2B} and A_3 ARs (A, B, C, D respectively) in chondrocyte membranes.

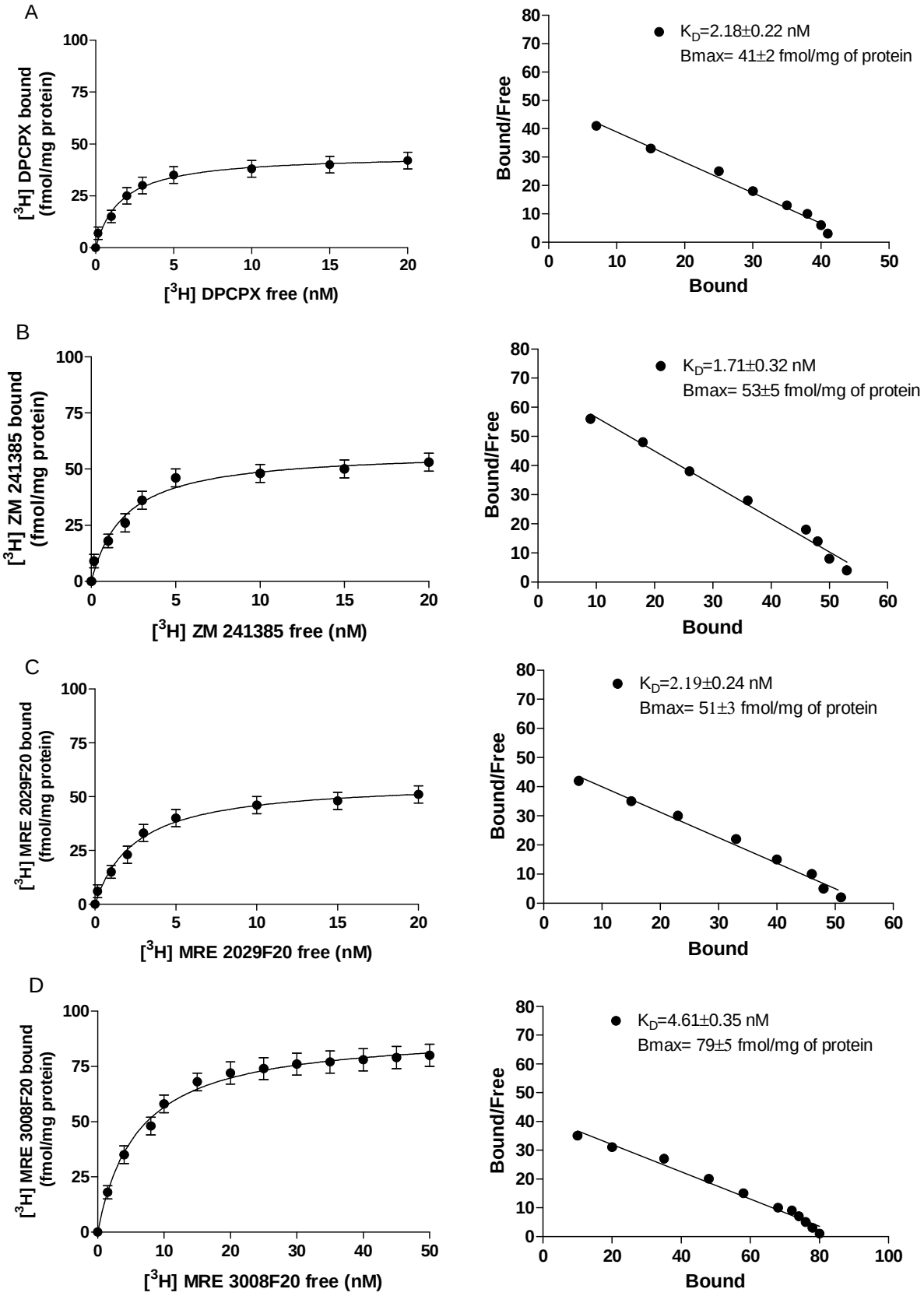


Figure 1.3: Saturation curves and Scatchard plots of A_1, A_{2A}, A_{2B} and A_3 ARs (A, B, C, D respectively) in fibroblast-like synoviocyte membranes.

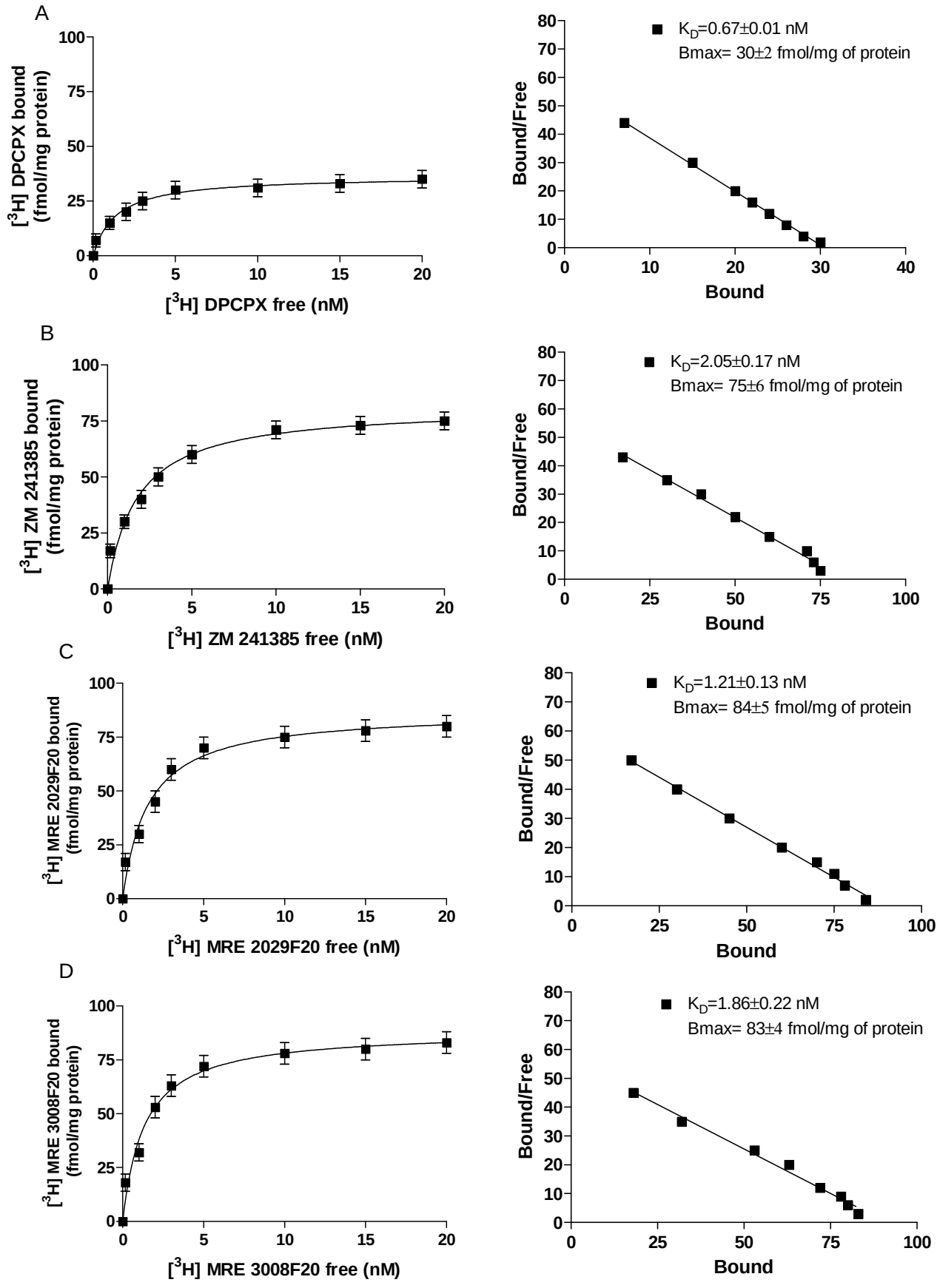


Figure 1.4: Competition curves of CGS 21680 (A) and Cl-IB-MECA (B) in bovine chondrocyte membranes.

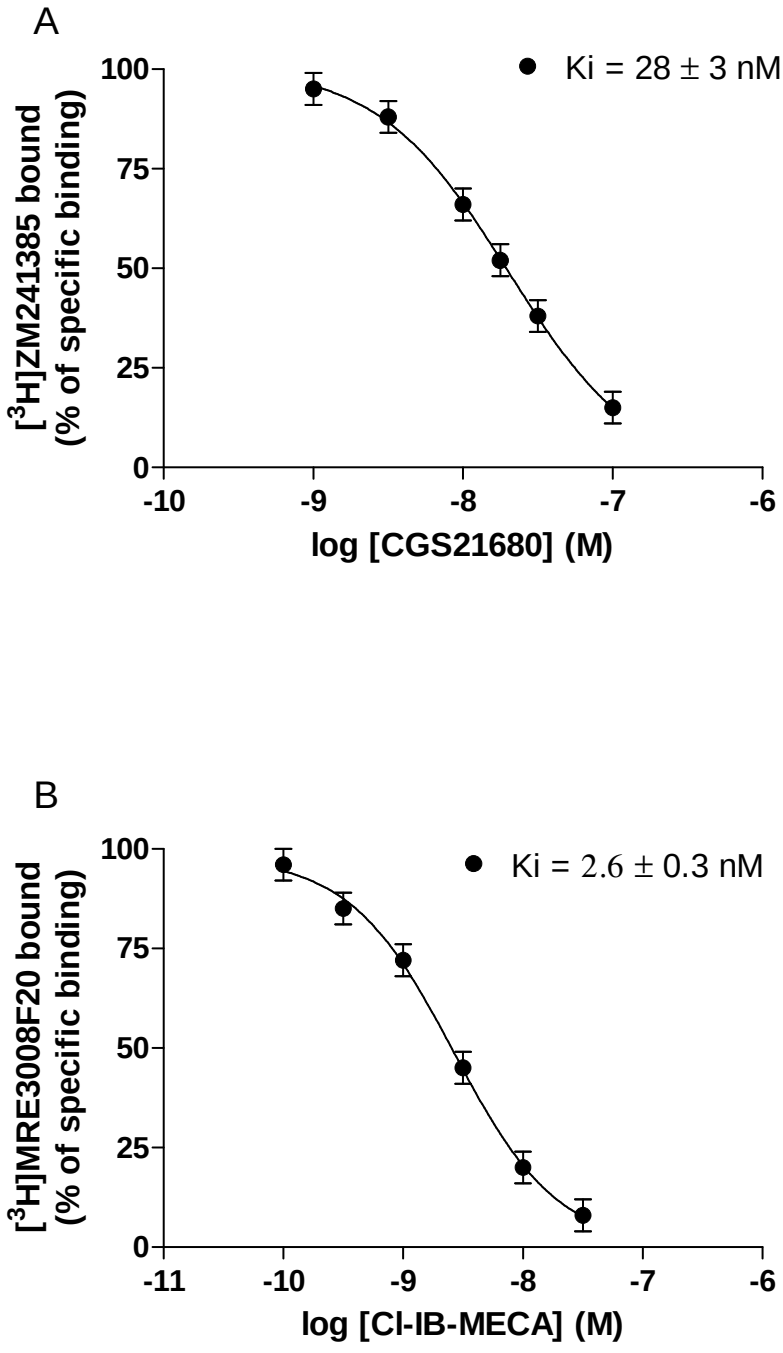


Figure 1.5: Competition curves of CGS 21680 (A) and Cl-IB-MECA (B) in bovine fibroblast-like synoviocyte membranes.

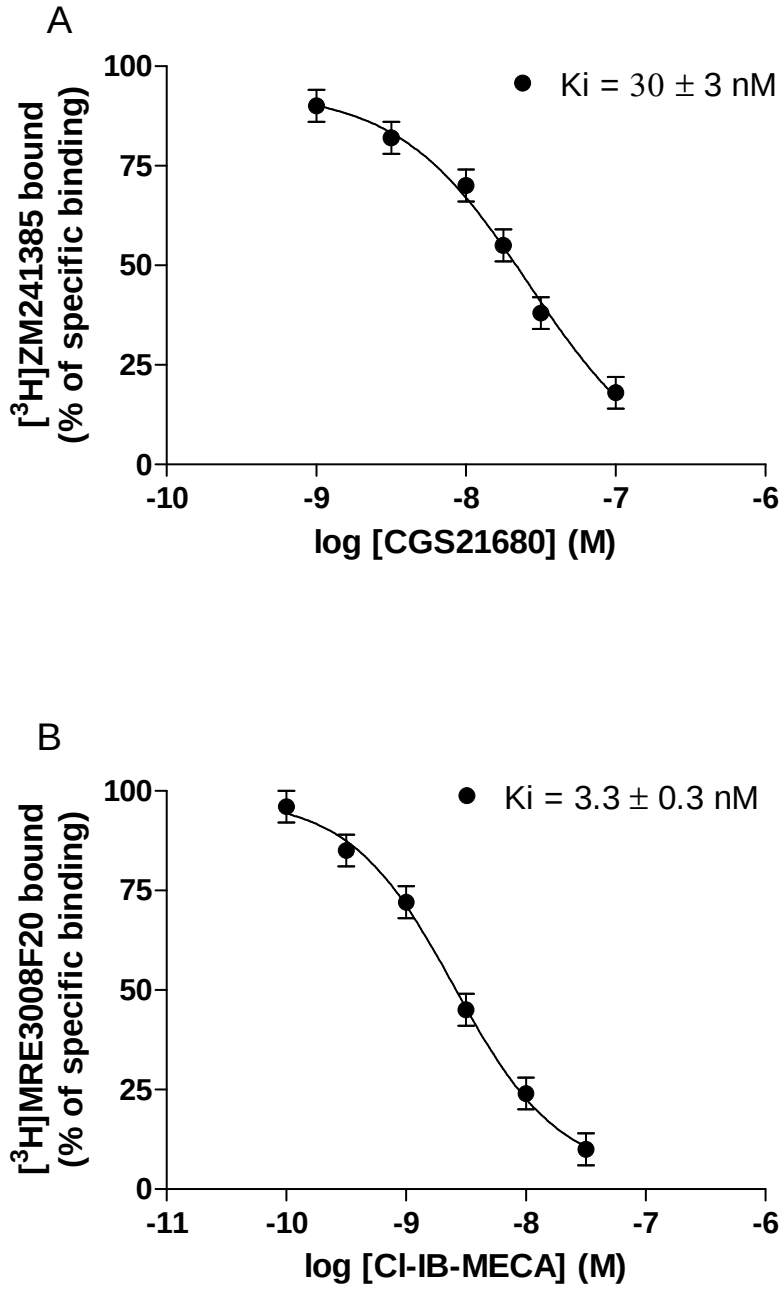


Figure 1.6: cAMP experiments by using CGS21680 (A) and Cl-IB-MECA (B) in bovine chondrocytes.

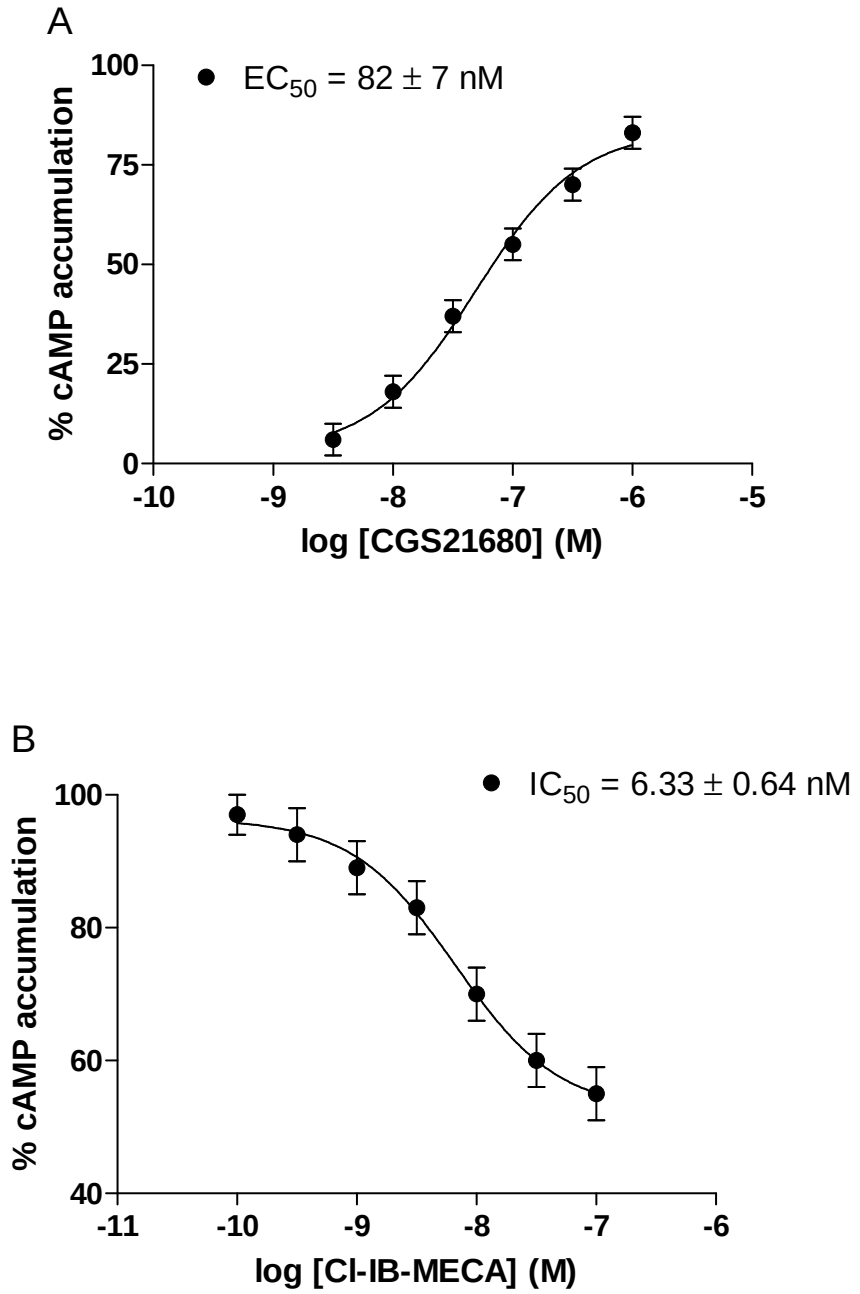


Figure 1.7: cAMP experiments by using CGS 21680 (A) and Cl-IB-MECA (B) in bovine fibroblast-like synoviocytes.

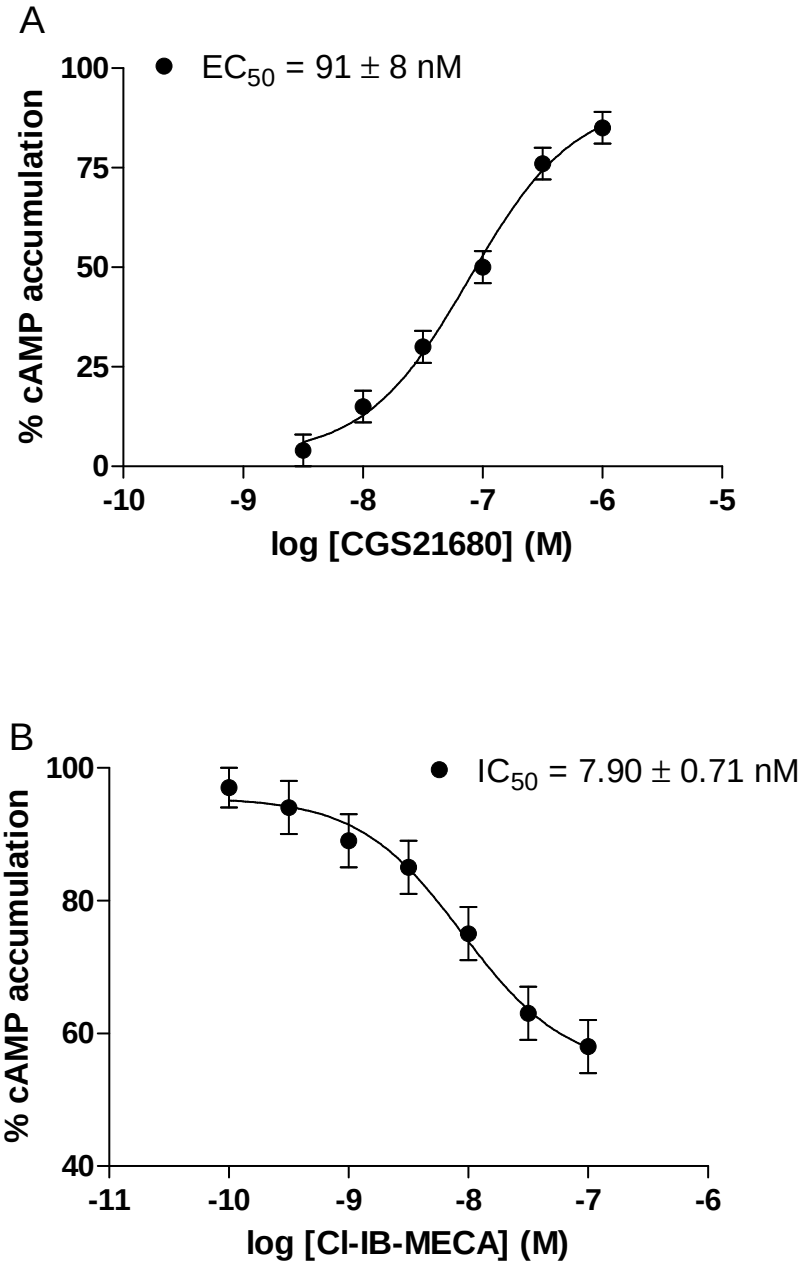
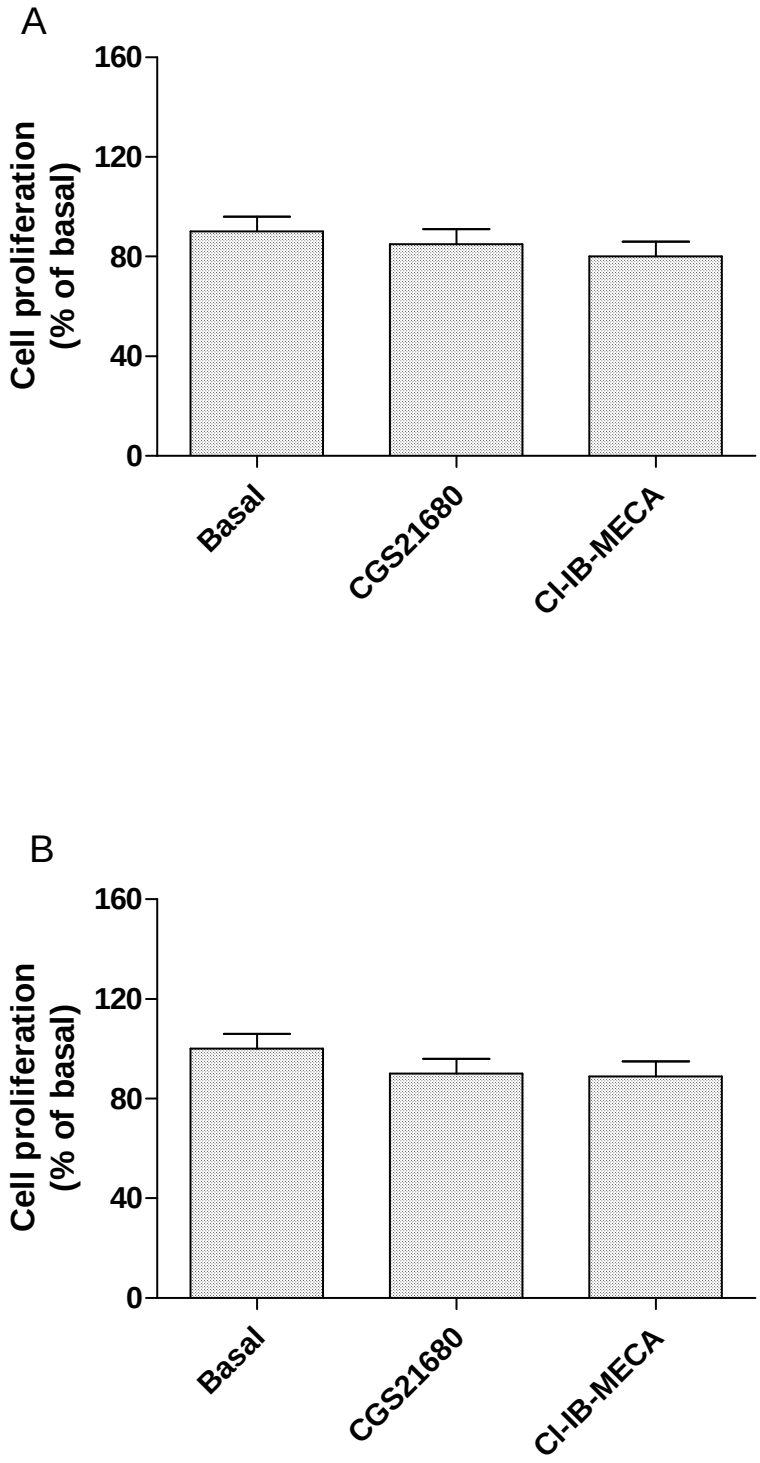


Figure 1.8: Proliferation activity measured by [^{3}H] thymidine incorporation assay in bovine chondrocytes (A) or fibroblast-like synoviocytes (B).



DISCUSSION

The purpose of the current investigation was to document the presence and the binding parameters of adenosine receptor subtypes in bovine chondrocytes or in fibroblast-like synoviocytes. Preliminary characterization of cells used in our experiments showed the expression of chondrocyte and synoviocyte phenotypic markers, as well as the absence of contaminating cells in fibroblast-like synoviocyte cultures (De Mattei et al., 2004). Our pharmacological data in these cells report that A_1, A_{2A}, A_{2B} and A_3 ARs are expressed and similarly distributed in both cell types showing high-affinity values in the nanomolar range and a receptor density from 30 to 83 fmol/mg of protein. The results obtained in competition binding experiments show that the affinity of CGS 21680 or Cl-IB-MECA in bovine chondrocytes were 28 ± 3 and 2.6 ± 0.3 nM respectively. In fibroblast-like synoviocytes the affinity of CGS 21680 was 30 ± 3 nM and for Cl-IB-MECA was 3.3 ± 0.3 nM. The effect of A_{2A} and A_3 agonists on adenylyl cyclase activity and cell proliferation was investigated. Our results did not show any change of basal enzyme activity and of the response of adenylyl cyclase to the direct activator forskolin used in the absence or in the presence of cAMP-dependent phosphodiesterase inhibitor (Ro 20-1724). Forskolin, which directly activated adenylate cyclase was utilized in this study as a positive control for cAMP production. Ro 20-1724, a type IV phosphodiesterase inhibitor prevented the rapid degradation of cAMP allowing the accurate detection of the cAMP levels produced. Moreover, the capability of typical A_{2A} or A_3 adenosine agonists such as CGS 21680 or Cl-IB-MECA to modulate cAMP levels was evaluated. These compounds showed potency values in the nanomolar range, in agreement with their affinity in binding experiments. To further confirm that the effects induced by the agonists on cAMP formation were due to the modulation of adenosine receptors investigated, we performed experiments in the presence of typical selective A_{2A} or A_3 adenosine antagonists such as SCH 58261 or MRE 3008F20. These antagonists were able to prevent the effect of cAMP induced by CGS21680 or Cl-IB-MECA through a selective modulation of the adenylyl cyclase via the A_{2A} or A_3 ARs, respectively. In addition, the levels of cAMP increased in response to NECA, a nonselective agonist, suggesting that in physiological condition (presence of endogenous adenosine) the activation of A_{2A}

ARs may prevail over the A_3 ARs inhibitory action. This is not surprising because a similar behavior has already been reported in other substrates (Gessi et al., 2002, 2004). In line with cAMP data, cell proliferation in the absence of adenosine agonists and in the presence of ADA was not modify even if Cl-IB-MECA inhibited slightly cell proliferation in fibroblast-like synoviocytes. In conclusion, this study shown for the first time the presence and the binding parameters of the A_1, A_{2A}, A_{2B} and A_3 ARs, in bovine chondrocytes and fibroblast-like synoviocytes. Functional assays performed on A_{2A} and A_3 ARs showed the involvement of these subtypes in bovine chondrocytes and fibroblast-like synoviocytes. These results open interesting perspectives on the modulation of adenosine pathways for the treatment of joint inflammatory diseases.

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CHAPTER 2:

Adenosine analogs inhibit prostaglandin E_2 (PGE_2) release in bovine fibroblast-like synoviocytes.

INTRODUCTION

OA and RA are the most common degenerative diseases of the joints, characterized by the progressive and permanent degradation of the articular cartilage, synovial hyperthrophy and change in underlying bone. Although the pathophysiologic events of OA and RA are quite different, inflammation and altered fibroblasts-like synoviocytes activities are observed in affected joints (Pelletier and Martel-Pelletier, 2007; Goldring, 2007; Muller-Landner et al., 2005; Moulton, 1996; Christodoulou and Choy, 2006; Abeles and Pillinger, 2006). In RA, fibroblasts-like synoviocytes play a central role to the pathogenesis of joint destruction by an increased proliferation and the secretion of a wide range of proinflammatory mediators, including cytokines, growth factors, and lipid mediators of inflammation (Muller-Landner et al., 2005; Abeles and Pillinger, 2006). Pro-inflammatory mediators produced by fibroblasts-like synoviocytes are detrimental to articular cartilage also in OA, although fibroblasts-like synoviocytes play a less central role than in RA (Pelletier and Martel-Pelletier, 2007; Goldring, 2007). Inflammatory mediators released by SFs can impact several cell types including lymphocytes, neutrophils, endothelial cells, osteoclasts and chondrocytes and a range of processes including maintenance of inflammation, angiogenesis, chemoattraction. Furthermore, fibroblasts-like synoviocytes are involved in cartilage destruction via the synthesis and secretion of matrix degrading enzymes such as matrix metalloproteinases (MMPs) and aggrecanases (Asano et al., 2006; Westra et al., 2004; Davidson et al., 2006). Prostaglandins, in particular PGE_2, are important lipid inflammatory mediators produced by fibroblasts-like synoviocytes and their levels are increased in the synovial fluid and synovial membrane of patients with inflammatory joint diseases (Park et al., 2006; Burger et al., 2003; Fahmi et al., 2001; Goetzl et al., 1995). PGE_2 is synthesized from arachidonic acid via the actions of cyclooxygenase (COX) enzymes, either constitutively or in response to cell specific trauma, stimuli, or signalling molecules such as IL-1β or TNF-α and it accounts for many of the proinflammatory actions induced by these peptides (Berenbaum, 2000; Funk, 2001; Crofford, 1999). PGE_2 contributes to vasodilatation, vascular permeability, pain, cytokine and proteinase production in inflamed tissues (Hill et al., 2001; Cha et al., 2004; Agro et al., 1996).

Accordingly, the use of non-steroidal anti-inflammatory drugs (NSAIDs) and/or selective COX-2 inhibitors which control inflammation and inhibit PGE_2 production represent the standard recommended treatment of OA and RA (Schnitzer, 2001; Hochberg et al., 1995). Adenosine, interacting with four types of cell surface receptor (AR) proteins, termed A_1, A_{2A}, A_{2B} and A_3 acts as a potent endogenous inhibitor of inflammatory processes in several tissues (Fredholm et al., 2001; Varani et al., 2003a; Gessi et al., 2002, 2004). In fibroblasts-like synoviocytes and chondrocytes adenosine has been involved in the production of matrix metalloproteinases (MMPs) and inflammatory mediators (Tesch et al., 2002, 2004). Moreover, in human fibroblasts-like synoviocytes the selective involvement of the A_{2A} AR subtype in the immunomodulatory actions of methotrexate has been reported (Ralph et al., 2005). Further, in vivo adenosine analogs inhibit joint destruction when used in the treatment of adjuvant induced arthritis and septic arthrosis (Boyle et al., 2002). On the bases of the above observations and of the role of fibroblasts-like synoviocytes to elicit and to maintain joint inflammation, we investigated if adenosine receptor agonists, might limit PGE_2 release in fibroblasts-like synoviocytes treated with known inflammatory stimuli: TNF-α and the bacterial lipopolysaccharide (LPS) (Westra et al., 2004; Park et al., 2004). COX-2 expression was also evaluated by RT-PCR as it appears to be the primary enzyme controlling PGE_2 synthesis in response to inflammatory stimuli (Park et al., 2006; Crofford, 1999). Further, adenosine analogs were used to characterise the presence of adenosine receptors and their affinity in bovine fibroblasts-like synoviocytes.

MATERIALS AND METHODS

Fibroblasts-like synoviocytes cultures

Fibroblasts-like synoviocytes were obtained by culture of the bovine synovial fluid, aspirated from the metacarpophalangeal joints of 14-18 month-old animals, as previously described (Varani et al., 2007). Fibroblasts-like synoviocytes at the third-fourth passage were used in the experiments. Cells were characterized by immunofluorescence staining with vimentin, a marker for fibroblasts (Upragarin et al., 2005). To confirm that SF cultures were not contaminated by macrophages, CD14 expression was evaluated by RT-PCR.

Immunofluorescence staining

Fibroblasts-like synoviocytes were fixed with cold methanol, washed with phosphate-buffered saline (PBS) and incubated with the primary monoclonal antibody (mAb) for the human vimentin (SigmaeAldrich, Italy) at 1:200 dilution for 1 h at 37°C. Washed slides were then incubated with a secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibody for 1 h at 37°C. Nuclei were stained with the DNA dye, 4',6-diamidino-2-phenylindole (DAPI) (0.1 mg/ml in PBS ethylene glycol tetraacetic acid (EGTA)) for 10 min. Fluorescence was visualized using the Nikon Eclipse TE 2000-E microscope (Nikon Instruments, Italy) equipped with a digital camera (DXM 1200F).

RT-PCR experiments

CD14 and COX-2 expression in fibroblasts-like synoviocytes cultures was assayed by RT-PCR. Total RNA extraction was performed by a commercial kit (RNeasy Kit, Qiagen, Deutschland). RNA conversion to cDNA was performed by the kit SuperscriptTM First-Strand Synthesis System (Invitrogen, USA). Oligonucleotide primers for CD14 were dp50'-CTGGAAGCCGGCG-3'; rp5'-AGCTGAGCAGGAACCTGTGC-3' and oligonucleotides for glyceraldehydes 3-phosphate dehydrogenase (GAPDH) were dp5'-TGGCAT CGTGGAGGGACTTAT-3'; rp5'-GACTTCAACAGCGACACTCAC-3'. Sequences were selected to amplify both human and bovine genome. Oligonucleotides for COX-2 were dp5'-TCCAGATCACATTTGATTGACA-3'; rp5'-TCTTTGACTGTGGGAGGATACA-3'. Oligonucleotides sequences were from separate exons to exclude genomic DNA contaminations. Two microliters of cDNA were amplified by the specific oligonucleotide sets and PCR reactions were performed as previously described (Varani et al., 2007). Cycling parameters were as follows: 1 min at 94°C; 1 min at the specific annealing temperature (55°C for CD14, 61°C for GAPDH, 60°C for COX-2); and 1 min at 72°C. PCR product sizes were 403 bp for CD14, 370 bp for GAPDH and 450 bp for COX-2. mRNA from human macrophages was used as a positive control for CD14 expression. PCR products were analyzed on 1.5% agarose gel, stained with ethidium bromide and visualized under UV.

Saturation and competition binding experiments to ARs

Fibroblasts-like synoviocytes, used for cellular membrane preparations, were washed with PBS and scraped off T75 flasks in ice-cold hypotonic buffer (5 mMTris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized by a Polytron, centrifuged for 30 min at 100,000g and used in saturation and competition binding experiments (Varani et al., 2007, 2002, 2003a,b). Saturation experiments to A_1 ARs were performed using [^{3}H]-N6-cyclohexyladenosine ([^{3}H]-CHA, 34.4 Ci/mmol; NEN - Perkin Elmer, USA) as radioligand for an incubation time of 90 min. Non-specific binding was determined in the presence of CHA 1 μM. Saturation experiments to A_{2A} ARs were performed using [^{3}H]-CGS 21680 (39.6 Ci/mmol; NEN - Perkin Elmer, USA) as radioligand with an incubation time of 120 min. Non-specific binding was determined with CGS 21680 1 μM. Saturation experiments to A_{2B} ARs were performed using [^{3}H]-MRE 2029F20, (123 Ci/mmol; Amersham Laboratories, UK) as radioligand for 60 min. Non-specific binding was determined with MRE 2029F20 1 μM. Saturation experiments to A_3 ARs were performed using [^{125}I]-4-aminobenzyl-50-N-methylcarboxamidoadenosine ([^{125}I]-AB-MECA, 2000 Ci/mmol; Amersham Laboratories, UK) as radioligand, for 60 min (Varani et al., 1998). Non-specific binding was determined with AB-MECA 1 μM. In competition experiments, carried out to determine A_1, A_{2A}, A_{2B} and A_3 affinity values, 1 nM radioligands were used on fibroblasts-like synoviocytes membranes (100 mg protein per assay) and 6-8 different concentrations of the examined agonists. At the end of incubation time, in binding experiments performed in fibroblasts-like synoviocytes membranes, bound and free radioactivities were separated by filtering the assay mixture through Whatman GF/B filters (Brandel Harvester) and radioactivity was counted (Scintillation Counter Packard Tri Carb 2500TR).

Fibroblasts-like synoviocytes treatments with adenosine agonists

For the analysis of PGE_2 release, fibroblasts-like synoviocytes at third-fourth passage were plated at 10,000/cm^2 in complete medium DMEM/F12 containing 10% FBS and antibiotics in multiwells (Nunc, Denmark, 1.6 cm the diameter of each well) and used after 5 days plating. In preliminary experiments increasing doses of the recombinant human TNF-α (Preprotech, USA) and the bacterial LPS (Sigma, USA) selected in the range of those used in previous studies, were tested.

In the following experiments TNF-α and LPS were used, respectively, at 10 ng/ml and 1 mg/ml, which elicited maximal PGE_2 increase in preliminary experiments. Control cells were incubated in complete medium alone. In a first series of experiments adenosine analogs were added to both control and TNF-α- or LPS-treated cultures in the presence of endogenous adenosine. The adenosine agonists CHA (A_1 AR), CGS 21680 (A_{2A} AR), NECA (non-selective), and Cl-IB-MECA (A_3 AR) were used at 1 μM (Sigma, USA). In a second series of experiments, treatments with adenosine agonists in the presence of TNF-α or LPS were performed in complete medium containing 2 IU/ml adenosine deaminase (ADA, FlukaeSigmae Aldrich, Switzerland) to deplete endogenously released adenosine. Different ADA concentrations (0.5-4 IU/ml) were analyzed on PGE_2 release and cell viability to evaluate the effect of endogenous adenosine. In some experiments, 1 μM forskolin (Sigma, USA), a direct activator of adenylate cyclase, was added to both control and TNF-α-treated cultures. At each condition tested, after 24 h treatment, medium was removed from the well, stored at -80°C for subsequent determination of PGE_2 and the monolayer protein content was evaluated (Lowry et al., 1951).

PGE_2 Assay

The concentration of PGE_2 was measured using a commercially available competitive enzyme immunoassay according to the manufacturer's instructions (PGE_2 ASSAY, R&D Systems, Inc. Minneapolis, USA). Samples and standards were assayed in duplicate. PGE_2 production was normalized to the total protein content and expressed as pg PGE_2/μg protein.

MTT assay

Effects of ADA on cell proliferation and viability of fibroblasts-like synoviocytes were evaluated by the MTT assay (Ahmed et al., 2006). Briefly, 100 μl of MTT solution (5 mg/ml in PBS) (Sigma-Aldrich, Irvine UK) were added to each well and incubated at 37°C for 3 h. The medium was then discarded and 500 μl of isopropanol/HCl 0.04 N were added to each well for the formazan solubilization. The solution absorbance was measured at 540 nm (Cary-50, UV-Visible Spectrophotometer, Varian).

Statistical Analysis

Data were obtained from at least five independent experiments. Each experiment was performed in triplicate. All values are expressed as mean ± SEM of four independent experiments. Analysis of data was done with Student's t test. Differences were considered significant at a value of $p<0.05$.

RESULTS

Phenotype characterization of fibroblasts-like synoviocytes

Fibroblasts-like synoviocytes used in our experiments are shown in Figure 2.1. Cells showed a fibroblast-like morphology and the expression of vimentin, the main intermediate filament protein in mesenchymal cells and fibroblasts-like synoviocytes. Results obtained by RT-PCR showed the absence of CD14 expression, indicating the absence of contaminating macrophages.

Evaluation of ARs affinity and density by saturation and competition binding experiments

Table 2.1 reports the affinity (K_D, nM) and density (Bmax, fmol/mg protein) of A_1, A_{2A}, A_{2B} and A_3 receptors in fibroblasts-like synoviocytes membranes. The affinity values of AR subtypes were in the low nanomolar range and the Bmax values were from 24 to 65 fmol/mg of protein. Table 2.2 reports the affinity values (Ki, nM) of CHA, NECA, CGS 21680 and Cl-IB-MECA in bovine fibroblasts-like synoviocytes. The major affinity of CHA (Ki = 3.5 ± 0.4 nM) was reported in [^{3}H]-CHA binding suggesting a high affinity vs A_1 AR. NECA and CGS 21680 showed similar affinity for A_{2A} AR even if NECA had a good affinity also for the other subtypes. Cl-IB-MECA showed a high affinity vs A_3 AR with a Ki value of 2.3 ± 0.2 nM. Saturation and competition binding experiments allowed to select, on the basis of the Ki values, the adenosine agonist concentrations to be used in subsequent experiments.

TNF-α and LPS induce a dose response increase on PGE_2 release in fibroblast-like synoviocytes

In preliminary experiments we investigated the effects of increasing doses of TNF-α and LPS, which are known to stimulate PGE_2 production in human

fibroblast-like synoviocytes, on PGE_2 release in bovine fibroblast-like synoviocytes cultures (Figure 2.2). PGE_2 production by control fibroblast-like synoviocytes was at very low levels. TNF-α and LPS significantly increased PGE_2 production in a dose-dependent manner. The most efficient doses were 10 ng/ml for TNF-α and 1 mg/ml for LPS yielding a maximal 7.9- and 9.8-fold increase, respectively. On these results, these TNF-α and LPS doses were used to stimulate PGE_2 production in subsequent experiments.

Adenosine agonist inhibit PGE_2 release in TNF-α- or LPS-treated fibroblast-like synoviocytes in the presence of endogenous adenosine

The effects of adenosine agonists on PGE_2 release in TNF-α or LPS unstimulated and stimulated fibroblast-like synoviocytes, in the presence of endogenous adenosine, are shown in figure 2.3. Treatment of cells with the A_1 agonist CHA, the A_{2A} agonist CGS 21680, the non-selective agonist NECA and the A_3 agonist Cl-IB-MECA did not modify basal PGE_2 production in the absence of TNF-α or LPS. In TNF-α stimulated fibroblast-like synoviocytes, all agonists, except for Cl-IBMECA, significantly inhibited PGE_2 production. PGE_2 inhibition ranged from 38.6% in the presence of CHA to 54.9% in the presence of NECA (Figure 2.3B). Similar data were obtained in LPS stimulated fibroblast-like synoviocytes (Figure 2.3C). The effects of adenosine agonists reduced PGE_2 levels to those of unstimulated control cells. Adenosine agonists were used at 1 μM, the concentration that assured a complete saturation of adenosine receptors as suggested from the affinity values (Ki, nM) of Table 2.2.

Depletion of endogenous adenosine with ADA increases basal PGE_2 release in fibroblast-like synoviocytes

Fibroblast-like synoviocytes were exposed to increasing doses of ADA (0.5-2 IU/ml) to determine the effects of depleting endogenous adenosine. PGE_2 release slightly but significantly increased in a dose-dependent manner with maximal effect at 2 IU/ml (Figure 2.4A). In parallel experiments, MTT assay was performed to verify that in our experimental conditions ADA did not modify fibroblast-like synoviocytes proliferation or viability. At all the doses, ADA had no effect on cell proliferation and viability (Figure 2.4B).

Depletion of endogenous adenosine with ADA potentiates adenosine agonist's effects on PGE_2 release in TNF-α- or LPS- treated fibroblast-like synoviocytes

As 2 IU/ml ADA induced the maximal increase in PGE_2 production, this dose was used to deplete endogenous adenosine (Figure 2.5). Both TNF-α and LPS significantly stimulated PGE_2 synthesis similarly to what observed in the absence of ADA. Also, Cl-IB-MECA did not modify PGE_2 levels in TNF-α or LPS stimulated cells. In TNF-α or LPS stimulated fibroblast-like synoviocytes, CHA, CGS 21680 and NECA induced a stronger inhibition on PGE_2 production than in the absence of ADA. In fact, CHA, CGS 21680 and NECA reduced PGE_2 levels to those of unstimulated control cells.

Forskolin stimulates PGE_2 release in activated fibroblast-like synoviocytes

As shown in figure 2.3 and 2.5, CHA and CGS 21680, which inhibit and stimulate adenylate cyclase, respectively, decreased PGE_2 release in TNF-α or LPS stimulated fibroblast-like synoviocytes. To clarify the potential involvement of adenylate cyclase activity in the modulation of PGE_2 production, we investigated the effects of forskolin, a potent stimulator of adenylate cyclase, on PGE_2 release. Forskolin did not modify basal PGE_2 levels. However, in fibroblast-like synoviocytes activated by the inflammatory stimuli, forskolin induced a further 24.3% increase in PGE_2 release.

Changes in COX-2 expression are associated to the changes in PGE_2 release induced by adenosine agonists in TNF-α- or LPS- treated fibroblast-like synoviocytes

Since PGE_2 levels were regulated by adenosine agonists we investigated whether changes in PGE_2 release were associated to a regulation of COX-2 transcripts. COX-2 expression, evaluated by RT-PCR, at 24 h treatment, is shown in figure 2.6. As reported in literature, in our experiments the stimulation of PGE_2 synthesis induced by TNFα and LPS was associated to an increase of COX-2 expression with respect to control cells. All adenosine agonists, except for Cl-IB-MECA, inhibited COX-2 expression in TNF-α or LPS stimulated cells.

Table 2.1 Affinity and density of adenosine receptors in bovine fibroblast-like synoviocytes.

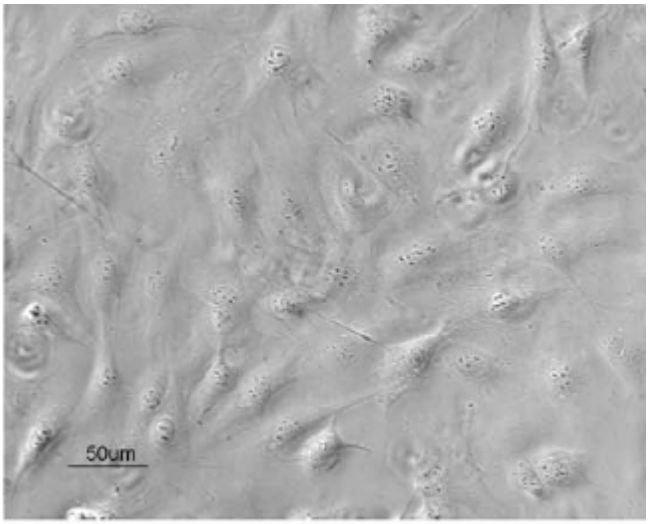
[^{3}H]CHA binding to A_1 ARs	[^{3}H]CGS 21680 binding to A_{2A} ARs	[^{3}H]MRE 2029F20 binding to A_{2B} ARs	[^{3}H]AB-MECA binding to A_3 ARs
K_D= 1.82 ± 0.16 nM Bmax= 24 ± 3 fmol/mg protein	K_D= 4.6 ± 0.5 nM Bmax= 62 ± 6 fmol/mg protein	K_D= 1.32 ± 0.12 nM Bmax= 78 ± 8 fmol/mg protein	K_D= 1.65 ± 0.15 nM Bmax= 65 ± 6 fmol/mg protein

Table 2.2 Affinity (Ki) of typical adenosine agonists versus A_1, A_{2A}, A_{2B} and A_3 ARs, in bovine fibroblast-like synoviocytes.

	[^{3}H]CHA binding to A_1 ARs Ki (nM)	[^{3}H]CGS 21680 binding to A_{2A} ARs Ki (nM)	[^{3}H]MRE 2029F20 binding to A_{2B} ARs Ki (nM)	[^{3}H]AB-MECA binding to A_3 ARs Ki (nM)
CHA	3.5 ± 0.4	812 ± 75	> 1000	83 ± 7
NECA	22 ± 2	8.4 ± 0.8	156 ± 17	36 ± 4
CGS21680	740 ± 70	7.2 ± 0.7	> 1000	985 ± 90
Cl-IB-MECA	185 ± 22	643 ± 60	> 1000	2.3 ± 0.2

Figure 2.1: Bovine fibroblast-like synoviocytes in culture. Phase contrast (A) and vimentin expression by immunofluorescence (B) nuclei were counterstained in blue with DAPI.

A



B

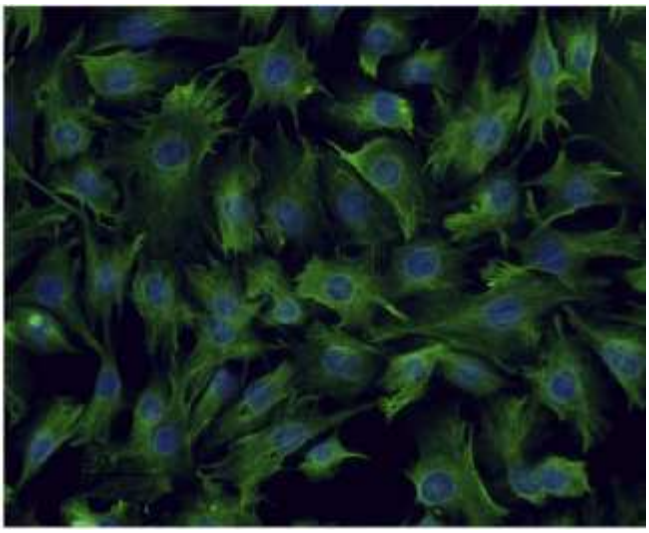
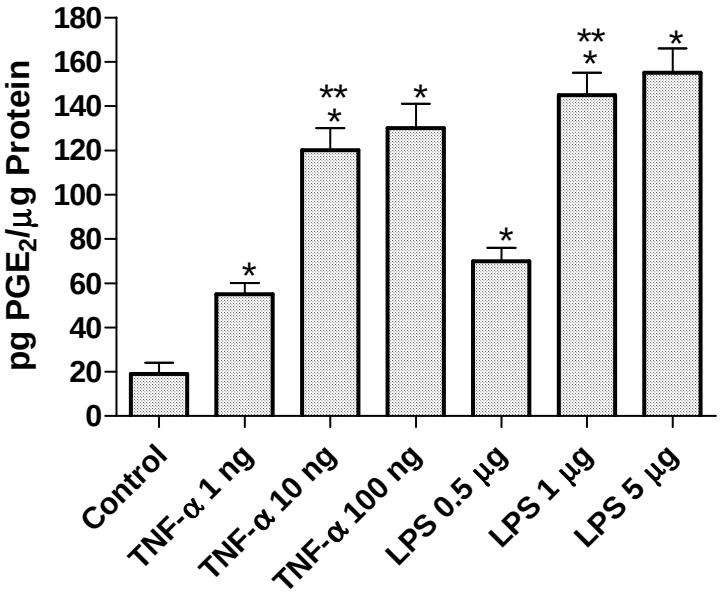


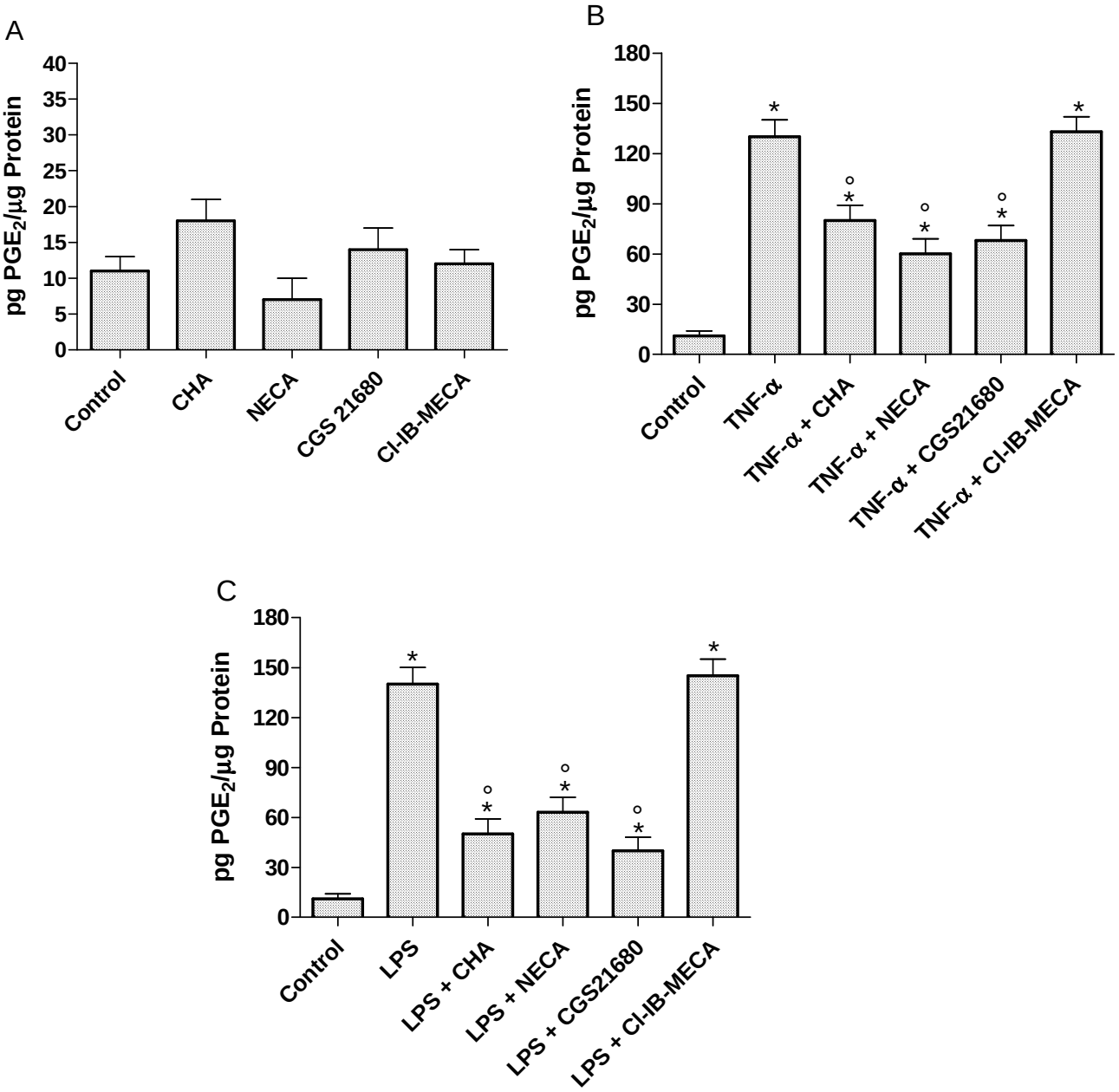
Figure 2.2: Effect of increasing doses of TNF-α and LPS on PGE_2 production in bovine fibroblast-like synoviocytes.



* Indicates statistical significance ($p<0.05$) vs control.

** Indicates statistical significance ($p<0.05$) vs the previous dose.

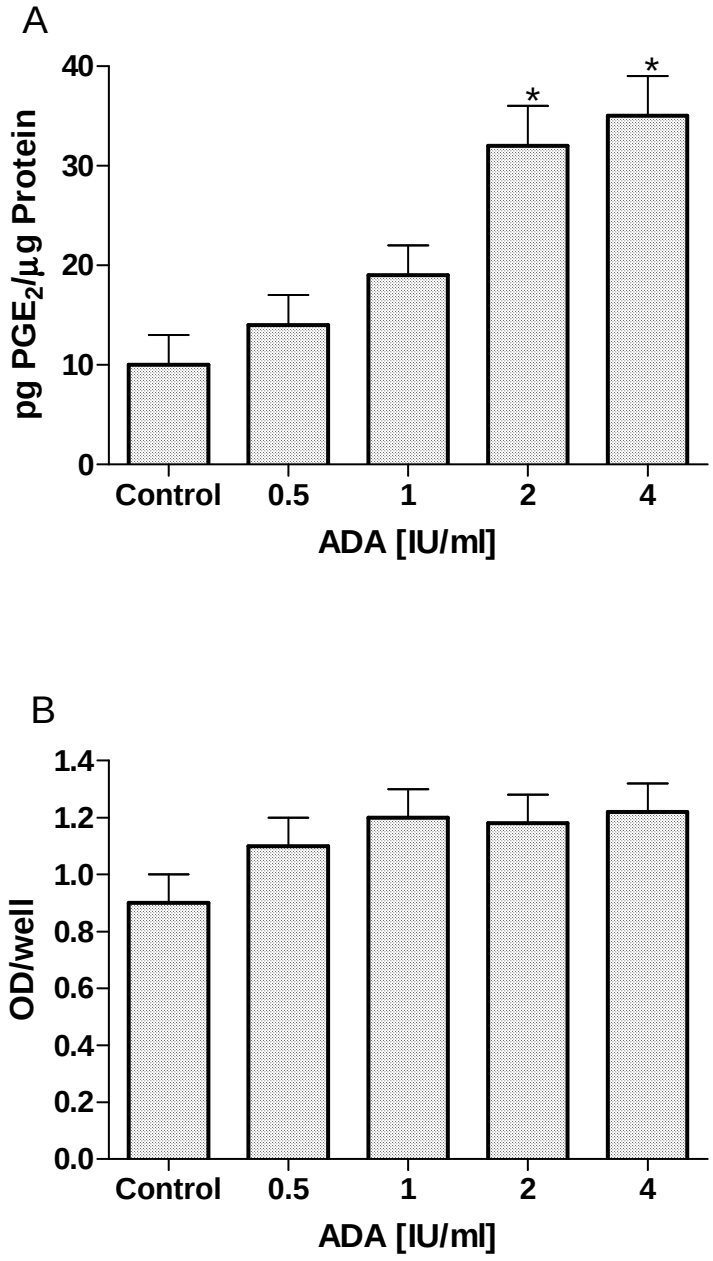
Figure 2.3: Effect of AR agonists, in the presence of endogenous adenosine, on basal (A), TNF-α (B) or LPS (C) induced PGE_2 production in bovine fibroblast-like synoviocytes.



* Indicates statistical significance ($p<0.05$) vs control.

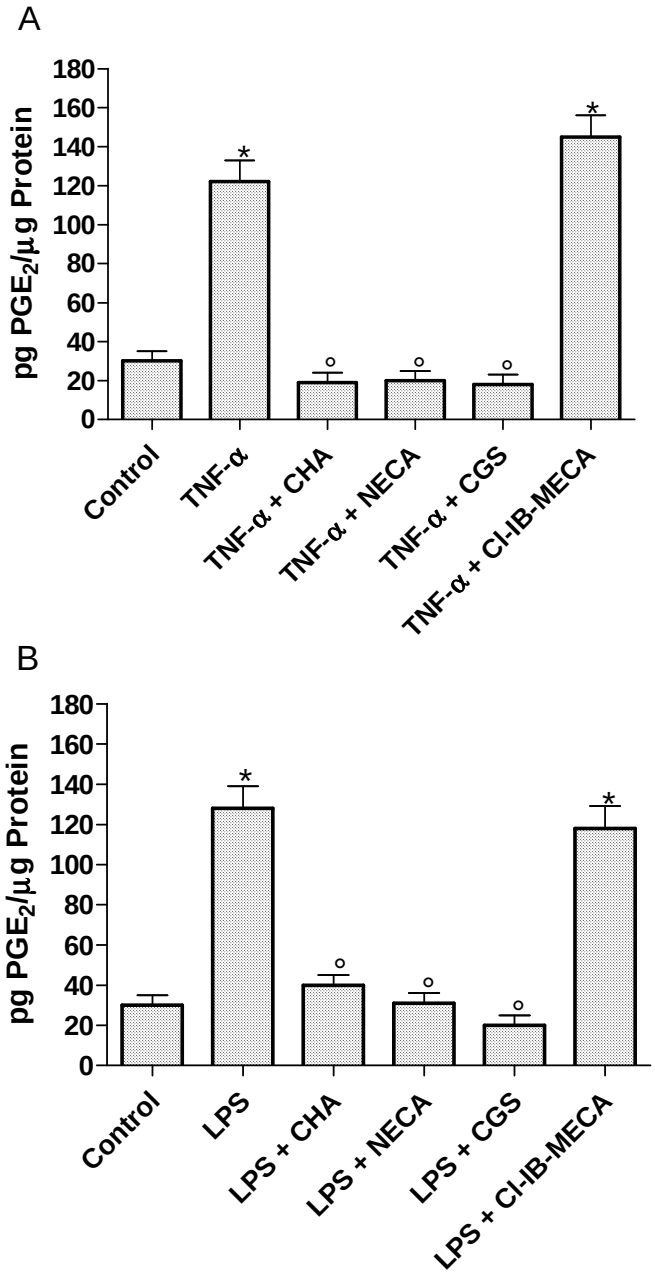
° Indicates statistical significance ($p<0.05$) vs inflammatory stimuli (TNF-α 10 ng/ml, LPS 1 µg/ml).

Figure 2.4: Effect of increasing doses of ADA on PGE_2 production (A) and cell proliferation/viability evaluated by MTT test (B).



* Indicates statistical significance ($p<0.05$) vs control.

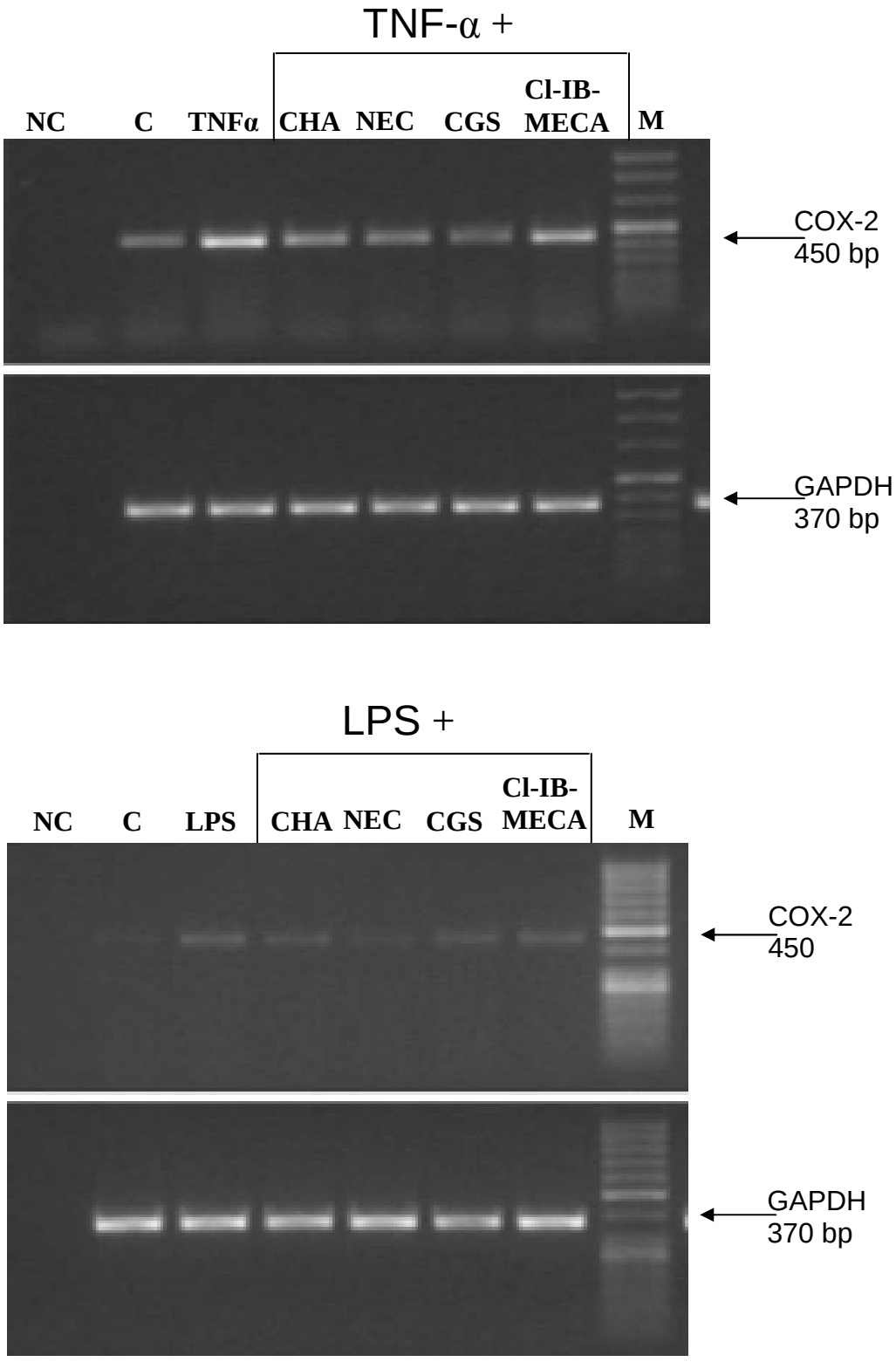
Figure 2.5: Effect of adenosine agonists, in the presence of ADA (2 IU/ml), on TNF-α (A) or LPS (B) induced PGE_2 production in bovine fibroblast-like synoviocytes.



* Indicates statistical significance ($p<0.05$) vs control.

° Indicates statistical significance ($p<0.05$) vs inflammatory stimuli (TNF-α 10 ng/ml, LPS 1 µg/ml).

Figure 2.6: Effect of adenosine agonists on control and TNF-α (upper panel) or LPS (lower panel) induced COX-2 expression.



DISCUSSION

In this study we investigated how AR agonists, might modify PGE_2 release in bovine fibroblast-like synoviocytes treated with pro-inflammatory stimuli. Saturation binding experiments confirmed the presence of A_1, A_{2A}, A_{2B} and A_3 ARs in fibroblast-like synoviocytes (Varani et al., 2007, 2002). In agreement with previous studies, TNF-α and LPS induced an approximately 8-10-fold increase in PGE_2 levels in fibroblast-like synoviocytes. In a first series of experiments we analyzed the effects of adenosine agonists in the presence of endogenous adenosine. All the agonists had no effect on basal PGE_2 release, however, CHA, NECA and CGS 21680 caused a significant inhibition on PGE_2 increase induced by TNF-α and LPS. These data indicate for the first time the involvement of A_1 and A_2 ARs in the negative modulation of PGE_2 synthesis in fibroblast-like synoviocytes. Cl-IB-MECA did not modify PGE_2 production suggesting that the activation of the A_3 AR is not involved in the modulation of PGE_2 synthesis. As endogenous adenosine potentially could mask the selective involvement of a specific AR, we investigated the agonist effects also in the presence of ADA, an enzyme capable to deplete adenosine levels by its ability to convert adenosine to inosine (Tesh et al., 2004). The presence of ADA increased basal PGE_2 levels, confirming the involvement of adenosine in modulating PGE_2 production, a finding consistent with the first series of experiments and with previous studies in cartilage cells (Petrov et al., 2005). Further, in cells treated with TNF-α and LPS, CHA, NECA and CGS 21680 induced a stronger PGE_2 inhibition than in the absence of ADA; this can be explained by the increased potency of adenosine agonists in comparison to adenosine. Collectively, our results show that A_1 and A_{2A} ARs are involved in the inhibition of PGE_2 production in fibroblast-like synoviocytes. The similar effects on PGE_2 release obtained by using NECA (non-selective agonist) and CGS 21680 (A_{2A} AR agonist) suggest that A_{2B} AR are not involved in this functional response. In addition, the lack of an effect of Cl-IB-MECA excludes a role for the A_3 AR in modulating PGE_2 production. The canonical transduction pathway coupled to A_1 and A_{2A} ARs include, respectively, the inhibition and the stimulation of adenylate cyclase with consequent reduction and increase in cAMP levels. As both A_1 and A_{2A} agonists inhibited PGE_2 release to a similar degree, this suggested that cAMP changes were not involved in the

PGE_2 inhibition. Therefore, as a positive control for the adenylate cyclase activation, we investigated the effects of forskolin, a direct activator of this enzyme, on PGE_2 release. Forskolin increased PGE_2 release in stimulated fibroblast-like synoviocytes confirming that the PGE_2 release inhibition observed in our experiments was not linked to cAMP production; these findings are consistent with the results described by Kojima and co-workers (Kojima et al., 2003) in human fibroblast-like synoviocytes. Other signal transduction pathways, activated by ARs, might be involved in the negative regulation of PGE_2 production (Schulte and Fredholm, 2003; Ciccarelli et al., 2007). Finally, our results show that the ability of adenosine agonists to inhibit PGE_2 release is mediated by a down-regulation of TNF-α - and LPS-induced COX-2 mRNA expression. Worth of note, a similar effect is also induced in human fibroblast-like synoviocytes by known anti-inflammatory drugs (Fahmi et al., 2001). The pharmacologic PGE_2 blockade by aspirin, NSAIDs and COX-2 inhibitors has been a useful anti-inflammatory strategy for more than a century, however, it is known that the appearance of side-effects may limit the chronic use of these drugs. The findings of the present study open new perspectives to the control of inflammation associated to joint diseases. It is to note that adenosine modulate chondrocyte activities too. In cartilage cells, adenosine and the A_{2A} AR have been involved in the inhibition of inflammatory and matrix degradative events (Tesh et al., 2002, 2004) promote anabolic activities and prevent cartilage degradation (De Mattei et al., 2003, 2004, 2007). Thus, previous observations and the results of this study suggest that adenosine analogs, may reduce inflammation and cartilage degradation in articular joints, by targeting both fibroblast-like synoviocytes and chondrocytes. Indeed, in vivo, the separate ability of adenosine analogs to limit joint destruction has been previously proven in animal models (Ciombor et al., 2003). In conclusion, our results add new relevant data in the analysis of adenosine anti-inflammatory activities by showing the involvement of A_1 and A_{2A} ARs in the inhibition of fibroblast-like synoviocytes responses to inflammatory stimuli. From a clinical point of view, the pharmacological modulation of adenosine pathways might have relevant therapeutic potential for the treatment of joint inflammatory diseases.

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CHAPTER 3:

Expression and functional role of adenosine receptors in regulating inflammatory responses in human fibroblast-like synoviocytes.

INTRODUCTION

Human fibroblast-like synoviocytes 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 synoviocytes are detrimental to articular cartilage in different joint diseases such as OA and RA (Abeles and Pillinger, 2006; De Mattei et al., 2009). OA is the most common form of arthritis, and is the single most important cause of disability in older adults (Benito et al., 2005; Goldring et al., 2007). At the present the current recommended treatment of OA involves weight loss, physical therapy and the use of pain relievers (Altman and Barkin, 2009). However these drugs do not reverse the degenerative process in OA and show some adverse effect on cartilage metabolism (Zhang et al., 2008). Adenosine is a modulator which interact with four cell surface receptor subtypes named as A_1, A_{2A}, A_{2B} and A_3 ARs which are coupled to different G-proteins (Burnstock, 2008). A_1 and A_3 ARs, through Gi proteins, mediate inhibition of the adenylate cyclase activity, whilst A_{2A} and A_{2B} ARs via Gs proteins stimulate cAMP production (Haskò et al., 2008). ARs modulation has an important role in the regulation of the inflammatory processes (Palmer and Trevethic, 2008; Ham and Rees, 2008; Gessi et al., 2008). Understanding how cytokine release is regulated endogenously can give important insight in various disease pathologies. It is well known that MAPKs like p38 are involved in controlling cellular responses as the release of pro-inflammatory cytokines (Fotheringham et al., 2004). The cell signaling pathways initiated by pro-inflammatory events converge on activation of the NF-kB which drive cytokines transcription and production (Wen et al., 2006). Notably, p38 MAPK is one of the kinases implicated in the phosphorylation of NF-kB inhibitors (IkBs) (Westra and Limburg, 2006). Once phosphorylated, IkBs undergo polyubiquitination and ultimately proteosomic degradation, allowing NF-kB to enter the nucleus and promote the transcription of inflammatory genes, such as TNF-α and IL-8 (Barnes and Karin, 1997). A role of adenosine in modulating bovine chondrocytes and fibroblast-like synoviocytes activity has been documented by previous studies of our group. In bovine fibroblast-like synoviocytes ARs have been characterized from saturation, competition binding experiments and western blotting analysis (Varani et al., 2008). Functional studies suggested an anti-inflammatory effect relative to A_1 and

A_{2A} ARs activation in LPS-induced PGE_2 production mediated by a down-regulation of TNF-α and COX-2 mRNA expression (De Mattei et al., 2009). It has been recently reported that in fibroblast-like synoviocytes from RA patients, A_3 ARs are over-expressed and an A_3 stimulation mediated a reduction in inflammation as a decrease in NF-kB and TNF-α release (Ochaion et al., 2008). Furthermore it has been demonstrated that in different cells or tissues adenosine is a regulator of NF-kB and MAPK signalling through the interaction with their receptor subtypes (Majumdar et al., 2003; Schulte and Fredholm, 2003; Jijon et al., 2005). From this background, the aim of this study was to investigate the presence of ARs in primary cultures of human fibroblast-like synoviocytes cells from OA patients by using mRNA and western blotting assays. Saturation binding experiments were performed to evaluate affinity (K_D) and density (Bmax) of A_1, A_{2A}, A_{2B} and A_3 ARs. Affinity values (Ki) of adenosine agonists and antagonists were determined by using competition binding experiments. In order to complete the pharmacological characterization, ARs were evaluated from a functional point of view. The effect of adenosine agonists and antagonists was investigated on cAMP production. Moreover, we focused the involvement of ARs on the signal transduction pathways including p38 MAPK and NF-kB. Consequently, the effect of adenosine agonists on TNF-α and IL-8 secretion were analyzed. The capability of adenosine antagonists to block the effect of the adenosine agonists was also carried out to better verify the involvement of ARs. Finally Gi, Gs and phosphatidylinositide-3-OH kinase (PI3K) pathways were investigated to study ARs signalling.

MATERIALS AND METHODS

Subjects

Synovial tissues were obtained from patients with end-stage OA undergoing total joint replacement surgery. 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. All patients (n=35, F/M: 23/12; age: 63.7 ± 3.4 yrs) enrolled in this study were recruited from the Department of Biomedical Sciences

and Advanced Therapies, Orthopaedic Clinic of the University Hospital of Ferrara, Italy. Each patient underwent medical history, physical examination, electrocardiogram and routine blood tests. Mean disease duration expressed as years ± SEM was 6 ± 1. Regarding pharmacological therapy of OA, 80% of the enrolled patients were receiving non steroidal anti-inflammatory drugs (NSAIDs) and 20% had no medications.

Cell culture

Primary lines of surface adherent fibroblast-like synoviocytes were isolated by enzymatic digestion of synovial tissues for 2-3 hours at 37°C in DMEM containing 1.5 mg/ml of collagenase type I-A and 1 mg/ml of hyaluronidase (Sigma-Aldrich, Milan, Italy). After digestion the cells have been recovered by centrifugation and plated in T25 culture flasks (Miyashita et al., 2004). Human fibroblast-like synoviocytes were maintained in culture in DMEM, 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mmol/L), passaged when reaching confluence and used at the 3rd-4th passages for binding and functional experiments. CHO or HEK 293 cells transfected with human A_1, A_{2A}, A_{2B} and A_3 ARs were prepared as previously described. Membrane preparation was performed prior of the competition binding experiments as previously described (Varani et al., 2000; 2005).

Human fibroblast-like synoviocyte characterization

Immunofluorescence with the primary monoclonal antibody specific for the human vimentin (Sigma Aldrich, St Louis, MO) was used to evaluate the expression of vimentin, a fibroblasts marker, in primary cultures of human fibroblast-like synoviocytes cells, as previously described. Nuclei were stained with the selective DNA dye, DAPI (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, Firenze, Italy). To exclude the presence of contaminating macrophages or endothelial cells, fibroblast-like synoviocyte cultures have been analyzed for CD14 and von-Willebrand factor (vWF) expression by reverse transcription polymerase chain reaction (RT-PCR). Briefly, 2 μl cDNA have been amplified by specific oligonucleotide primers for CD14 (for-5'-CTG GAA GCC GGC G-3'; rev5'-

AGC TGA GCA GGA ACC TGT GC-3') and for vWF (for-5'-TGG CCA GAC CTT GCT GAA GA-3'; rev-5'-CCA TTA TGG AGA ATC ACC TCC A-3'). Cycling parameters have been as follows: 1 min at 94°C; 1 min at the specific annealing temperature (62°C for CD14 and 55°C for vWF); and 1 min at 72°C. PCR product sizes are 405 bp for CD14 and 252 bp for vWF. mRNA from human macrophages and endothelial cells have been used as a positive control for CD14 and vWF expression respectively (Miyashita et al., 2004).

RT-PCR experiments

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assay (Gessi et al., 2004) of A_1, A_{2A}, A_{2B} and A_3 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, Warrington Cheshire, UK). For the real-time RT-PCR of A_1, $A_{2A,}$ A_{2B} and A_3 adenosine receptors the assays-on-demand™ Gene expression Products NM 000674, NM 000675, NM 000676 and NM 000677 were used respectively. For the real-time RT-PCR of the reference gene the endogenous control human GAPDH kits was used, and the probe was fluorescent-labeled with VIC™ (Applied Biosystems, Warrington Cheshire, UK). Genomic contamination was ruled out by including an RT-negative sample in each PCR set as a control.

Western blotting analysis

Human fibroblast-like synoviocytes were washed with ice-cold phosphate buffer saline containing sodium orthovanadate 1 mM, 4-(2-aminoethyl)-benzenesulfonyl fluoride 104 mM, aprotinin 0.08 mM, leupeptin 2 mM, bestatin 4 mM, pepstatin A 1.5 mM, E-64 1.4 mM (Sigma Aldrich, St Louis, MO). Cells were then lysed in Triton lysis buffer and the protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL). Aliquots of total protein sample (50 μg) were analyzed using antibodies specific for human A_1, $A_{2A,}$ A_{2B} and A_3 ARs (1 μg/ml dilution, Alpha Diagnostic, San Antonio, TX) and P-p38 (1 μg/ml dilution, Cell Signaling Technology, Danvers, MA) (Merighi et al., 2002). 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 (GE Healthcare, UK). Western blotting assays were also normalized against the housekeeping protein β-actin.

Saturation and competition binding experiments to ARs

To obtain membrane preparation the culture medium was removed and the cells were washed with PBS and scraped off T75 flasks in ice-cold hypotonic. The cell suspension was homogenized by using a Polytron, centrifuged for 30 min at 100000 g and used in the saturation and competition binding experiments. Saturation binding experiments on human fibroblast-like synoviocyte membranes were performed by using [^{3}H]-DPCPX, [^{3}H]-ZM241385, [^{3}H]-MRE 2029F20 and [^{3}H]-MRE 3008F20 as radioligands to study the presence of A_1, A_{2A}, A_{2B} and A_3 ARs, respectively. Briefly, these radioligands at different concentrations (0.01-20 nM or 0.01-30 nM) were incubated with 100 μg of protein per assay of membrane suspension for 90 min at 25°C (A_1 AR) or 60 min at 4°C (A_{2A} and A_{2B} ARs) or 150 min at 4°C (A_3 AR). Competition binding experiments of 1 nM [^{3}H]-DPCPX, [^{3}H]-ZM 241385, [^{3}H]-MRE 2029F20 and [^{3}H]-MRE 3008F20 were carried out to determine the A_1, A_{2A}, A_{2B} and A_3 ARs affinity values of the selected adenosine agonists and antagonists, respectively. In these assays human fibroblast-like synoviocyte membranes (100 μg protein per assay) were incubated with different concentrations of the examined agonists CHA, CGS 21680, NECA, Cl-IB-MECA and antagonists DPCPX, SCH 58261, MRE 2029F20 and MRE 3008F20. Non specific binding was determined in the presence of DPCPX, ZM 241385, MRE 2029F20 or MRE 3008F20 at the 1 μM concentration, respectively and was always < 25% of the total binding. Similar experiments were also performed by using A_1, A_{2A}, A_{2B} and A_3 adenosine agonists such as CHA, CGS 21680, NECA and Cl-IB-MECA at the 1 μM concentration, respectively and was always < 30% of the total binding. At the end of incubation time, bound and free radioactivity were separated by filtering, in a Brandel cell harvester, the assay mixture through Whatman GF/B glass-fiber filters. The filter bound radioactivity was counted in a liquid Scintillation Counter Tri Carb Packard 2500 TR (Perkin-Elmer Life and Analytical Sciences, Boston, USA). Similar competition binding experiments were performed in CHO cells transfected with human A_1, A_{2A} or A_3 ARs and

A_{2B}HEK 293 cells with the aim to evaluate affinity and selectivity of adenosine agonists and antagonists used in functional assays.

Measurement of cAMP levels

Human fibroblast-like synoviocytes (10^6 cells per sample) were suspended in 0.5 ml incubation mixture Krebs Ringer phosphate buffer, containing adenosine deaminase 1.0 IU/ml (Sigma, St Louis, MO) and preincubated for 10 min in a shaking bath at 37°C. Then the effect of selected adenosine agonists was studied by using CHA, CGS 21680, NECA and Cl-IB-MECA at 1 μM concentration. To better investigate the inhibitory effect of CHA and Cl-IB-MECA the cells were also incubated with forskolin (1 μM) and/or Ro 20-1724 (0.5 mM) as phosphodiesterase inhibitor. A_1, A_{2A}, A_{2B} and A_3 selected adenosine antagonists as DPCPX, SCH 58261 MRE 2029F20 and MRE 3008F20 at the 1 μM concentration were also used to verify the specific involvement of these subtypes in cAMP production. The final aqueous solution was tested to evaluate cAMP levels by using a competition protein binding assay with [^{3}H]-cAMP. At the end of the incubation time (150 min at 4°C) and after the addition of charchoal the samples were centrifuged at 2000 x g for 10 min and the clear supernatant was counted in a liquid Scintillation Counter Tri Carb Packard 2500 TR (Perkin-Elmer Life and Analytical Sciences, Boston, USA).

NF-kB activation

Nuclear extracts from human fibroblast-like synoviocytes were obtained by using a nuclear extract kit (Active Motif, Carlsbad, USA) following the manufacturer's instructions. The NF-kB activation was evaluated by detecting phosphorylated p50 and p65 proteins in nuclear extracts by using the TransAM NF-kB kit (Active Motif, Carlsbad, USA). Phosphorylated NF-kB subunits specifically bind to the immobilized oligonucleotides containing the NF-kB consensus site (5'-GGGACTTTCC-3'). The primary antibody used to detect NF-kB 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.

TNF-α and Il-8 release

TNF-α and Il-8 levels were measured in human fibroblast-like synoviocytes by using a highly sensitive enzyme linked immunosorbent assay (R and D Systems, Minneapolis, USA) in accordance with the manufacturer's instructions.

Data and statistical analysis

The protein concentration was determined according to a Bio-Rad method with bovine albumin as reference standard (Bradford MA, 1976). A weighted non linear least-squares curve fitting program Ligand was used for computer analysis of saturation and competition binding experiments (Munson and Rodbard, 1980). Functional experiments were calculated by non linear regression analysis using the equation for a sigmoid concentration-response curve (GraphPAD Prism, San Diego, CA, USA). Analysis of data was performed by repeated measures analysis of variance (ANOVA) followed by Bonferroni test that was used for multiple comparisons of data sets and was considered significant at a value of $p<0.02$. All data are reported as mean ± SEM of independent experiments. Each experiment was performed by using the fibroblast-like synoviocytes derived from one single donors, and was done in duplicate (for binding experiments) or in triplicate (for functional experiments). The experiments were repeated at least 3 or 4 times as indicated from n values that showed the number of patients used.

RESULTS

Phenotype characterization of human fibroblast-like synoviocytes

Cells isolated from synovial of OA patients showed to be a homogenous population as demonstrated by their fibroblast-like morphology (Figure 3.1A). Primary cultures of human fibroblast-like synoviocytes cells also showed the expression of vimentin, a specific cellular marker for mesenchymal cells and fibroblast-like synoviocytes (Figure 3.1B). RT-PCR data showed that, under our experimental conditions, mRNA for CD14 and vWF were not amplified, suggesting the absence of macrophage and endothelial cell contamination in fibroblast-like synoviocytes cell cultures (Figure 3.1C).

Evaluation of ARs mRNA expression and protein level

Figure 3.2A shows adenosine mRNA in human fibroblast-like synoviocytes by using real-time quantitative RT-PCR. The present analysis performed with primers specifically designed for the various cloned human ARs revealed the expression of A_1, A_{2A}, A_{2B} and A_3 mRNA. In particular, high levels of A_{2A} and A_3 mRNA were found in human fibroblast-like synoviocytes. The presence of ARs was also confirmed by western blot analysis (Figure 3.2B). In human fibroblast-like synoviocytes A_{2A} and A_3 ARs were present with a higher expression than A_1 and A_{2B} ARs as demonstrated by densitometric analysis reported in figure 3.2C.

Saturation and competition binding experiments

Saturation binding experiments in primary cultures of human fibroblast-like synoviocytes membranes were performed to evaluate affinity (K_D) and receptor density (Bmax) of ARs. Figure 3.3A illustrate saturation binding curves relative to A_1, A_{2A}, A_{2B} and A_3 ARs showing affinity in the nanomolar range and different receptor density. Scatchard plot analysis revealed the presence of an high affinity binding site as suggested by the linearity of the lines (Figure 3.3B). Computer analysis of the data failed to show a significantly better fit to a two site than to a one site binding model, indicating that in our experimental conditions is primarily present one class of high affinity binding site. Competition binding experiments to A_1, A_{2A}, A_{2B} and A_3 ARs by using selected adenosine agonists and antagonists in human fibroblast-like synoviocytes membranes were performed (Figure 3.4). As expected, CHA and Cl-IB-MECA showed biphasic competition binding curves versus A_1 and A_3 ARs, respectively as suggested by a significantly better fit to a two site binding model and by an Hill coefficient less than unity (0.54 and 0.63, respectively). Their competition binding curves were best described by the existence of one high affinity (K_H) and low affinity (K_L) agonist-receptor binding state (Figure 3.4A,D). Moreover, CGS 21680 and NECA agonists revealed good affinity values versus A_{2A} and A_{2B} ARs confirming a tight coupling between the receptors and G protein. For CGS 21680 and NECA, Hill coefficients were close to unity excluding the involvement of multiple coupling affinity states (Table 3.1). In addition, competition binding experiments were also carried out studying selected adenosine antagonists as DPCPX, SCH 58261, MRE 2029F20 and MRE 3008F20 revealing Ki values in the nanomolar range (Table 3.1). The affinity of

the adenosine agonists and antagonists versus human A_1, A_{2A}, A_{2B} and A_3 ARs expressed in CHO or HEK 293 cells was reported and compared with human fibroblast-like synoviocytes in Table 3.1. CHA showed an high affinity versus A_1 AR similarly to those obtained in human fibroblast-like synoviocytes. Our experimental data revealed a good affinity of CGS 21680 and NECA versus A_{2A} AR. Cl-IB-MECA presented a very high affinity versus human A_3 AR. DPCPX, SCH 58261, MRE 2029F20 and MRE3008F20 showed an high affinity versus A_1, A_{2A}, A_{2B} and A_3 ARs, respectively (Table 3.1). The high affinity in human fibroblast-like synoviocytes of these adenosine antagonists confirmed the binding with A_1, A_{2A}, A_{2B} and A_3 ARs, respectively (Table 3.1).

cAMP assays

The A_{2A} and A_{2B} ARs are coupled to stimulation of adenylate cyclase via Gs proteins which mediates an increase of cAMP production. CGS 21680 and NECA at the 1 µM concentration were able to mediate a significant increase in cAMP formation reaching 65-90 pmoles per 10^6 cells, respectively. The presence of a selective A_{2A} antagonist as SCH 58261 demonstrates a complete reduction in cAMP production (Figure 3.5A). MRE 2029F290 an A_{2B} adenosine antagonist, was only able partially to inhibit NECA-stimulated cAMP levels because of the dual effect of NECA as stimulatory agent for A_{2A} and A_{2B}ARs (Figure 3.5A). The effect of A_1 and A_3 agonists as CHA and Cl-IB-MECA (1 µM) was evaluated in the presence of 1 µM forskolin and Ro 20-1724 (0.5 mM). This experimental condition was chosen for the low basal levels (15-20 pmol cAMP per assay) which hamper the evaluation of a direct inhibitory effect. In our experimental conditions CHA and Cl-IB-MECA were able to decrease cAMP levels by 70%. DPCPX was not able to abrogate completely the inhibitory effect of CHA probably for A_3 AR activation. On the other hand, MRE 3008F20 blocked the inhibitory effect induced by the selective A_3 agonist.

p38 MAPK activation

Western blotting analysis showed that LPS (10 µg/ml) was able to increase phospho-p38 levels of 72% in comparison to basal levels. All adenosine agonists investigated at the 100 nM concentration were not able to significantly modify phospho-p38 levels (data not shown). Interestingly, at the 1 µM concentration

adenosine agonists such as CGS 21680 and NECA were able to inhibit significantly in a similar way the LPS-stimulated P-p38 levels (Figure 3.6A,B). Moreover, Cl-IB-MECA (1 μM) was also able to inhibit the LPS-stimulated P-p38 levels although in a minor extent (Figure 3.6A,B). SCH 58261 and MRE 3008F20 blocked the inhibitory effect of CGS 21680 and Cl-IB-MECA, respectively. On the contrary, MRE 2029F20 was not able to abrogate NECA effect probably due to the persistent A_{2A} and A_3 NECA modulation. CHA (1 μM) in the absence and in the presence of DPCPX (1 μM) had no effect in the modulation of P-p38 levels suggesting that A_1 AR were not implicated.

NF-kB activation in human fibroblast-like synoviocytes

NF-kB levels were evaluated studying p50 (Figure 3.7A) and p65 (Figure 3.7B) subunits activation. In primary cultures of human fibroblast-like synoviocytes, adenosine agonists examined were not able to decrease the LPS-stimulated NF-kB levels at a concentration of 100 nM with the exception of Cl-IB-MECA that mediated a reduction in p50 and p65 levels by 33 ± 3% and 24 ± 3%, respectively ($p<0.05$). All adenosine agonists investigated at the 1 μM concentration were able to inhibit the LPS-stimulated NF-kB levels. The inhibitory effect of CHA was reverted only by the A_3 antagonist MRE 3008F20. In addition, SCH 58261, but not MRE 3008F20, was able to block the reduction of NF-kB levels mediated by CGS 21680 because of the high or low affinity of this agonist for A_{2A} AR or A_3 AR, respectively (Table 3.1). The inhibitory effect of the adenosine agonist NECA was partially blocked ($p<0.05$) only by SCH 58261 and MRE 3008F20 suggesting the involvement of A_{2A} and A_3 ARs. Interestingly, the effect of NECA was completely abrogated by the simultaneous incubation with SCH 58261 and MRE 3008F20. The reduction in p50 and p65 activation mediated by Cl-IB-MECA was reverted only by MRE 3008F20 and not by SCH 58261. This effect was most likely due to the low affinity of the A_3 agonist, Cl-IB-MECA for A_{2A} AR (Table 3.1, Figure 3.7 A,B).

TNF-α and IL-8 production

In human fibroblast-like synoviocytes the effect of adenosine agonists and antagonists on TNF-α (Figure 3.8A) and IL-8 (Figure 3.8B) release was evaluated in the presence of LPS 10 μg/ml. Of the agonists examined at 100 nM

concentration only Cl-IB-MECA was able to decrease significantly TNF-α and IL-8 production by 35 ± 3% and 28 ± 2%, respectively ($p<0.05$). All adenosine agonists investigated at 1 µM concentration were able to significantly inhibit LPS-stimulated release of TNF-α and IL-8. The inhibitory effect of CHA was counteracted by MRE 3008F20 and not by DPCPX or SCH 58261 suggesting the involvement of A_3 ARs (Table 3.1). The effect of CGS 21680 was only blocked by SCH 58261 and not by MRE 3008F20 probably because of the low affinity of CGS 21680 for A_3 AR. NECA was able to significantly decrease the LPS-stimulated release of TNF-α and IL-8, an effect blocked by SCH 58261 or MRE 3008F20 ($p<0.05$), but not by DPCPX or MRE 2029F20. The simultaneous use of SCH 58261 and MRE 3008F20 completely reverted the inhibitory effect of NECA ($p<0.01$). Finally, only MRE 3008F20 was able to abrogate the effect of Cl-IB-MECA on TNF-α and IL-8 release.

Modulation of Gi, Gs and PI3K proteins

To determine whether the Gi pathway or phosphatidylinositide-3-OH kinase (PI3K) was involved in A_3 AR responses, human fibroblast-like synoviocytes were preincubated with the Gi inactivator pertussis toxin (100 ng/ml) for 2 h or with the PI3K inhibitor LY294002 (25 µM) for 20 min and then stimulated with Cl-IB-MECA (1 µM). The preincubation with pertussis toxin did not modify the Cl-IB-MECA-mediated inhibition of P-p38, NF-kB p50 or p65, TNF-α and IL-8 levels. In contrast, LY294002 incubation completely abolished the inhibition of P-p38, NF-kB p50 or p65, TNF-α and IL-8 levels by Cl-IB-MECA. These data suggest that the A_3 ARs signals through a PI3K pathway (Table 3.2). In order to verify whether the Gs pathway was involved in A_{2A} functional responses, human fibroblast-like synoviocytes were incubated with a direct activator of adenylyl cyclase activity, forskolin (1 µM). This compound was able to reduce P-p38, NF-kB p50 or p65, TNF-α and IL-8 levels suggesting the involvement of cAMP in A_{2A}-mediated responses (Table 3.2).

Table 3.1: Affinities expressed as pKi values of well-known adenosine agonists and antagonists in CHO or HEK 293 cells expressing human A_1, A_{2A}, A_{2B} or A_3ARs and in human fibroblast-like synoviocytes.

Compounds	*[^{3}H] DPCPX binding to hA_1CHO cells pK_H, pK_L or pKi*	*[^{3}H] ZM 241385 binding to hA_{2A}CHO cells pKi*	*[^{3}H] MRE 2029F20 binding to hA_{2B}HEK 293 cells pKi*	*[^{3}H] MRE 3008F20 binding to hA_3CHO cells pK_H, pK_L or pKi*	*Human synoviocytes*	
					pKi	n_H
CHA	8.96 ± 0.04 6.76 ± 0.03	6.09 ± 0.03	4.38 ± 0.02	7.19 ± 0.05 5.57 ± 0.04	9.08 ± 0.03[a] 6.94 ± 0.04	0.54±0.05
CGS 21680	> 5.3	7.92 ± 0.03	> 5.3	5.91 ± 0.04	7.55 ± 0.05[b]	1.04±0.09
NECA	7.70 ± 0.04	8.08 ± 0.04	6.74 ± 0.03	7.44 ± 0.05	7.02 ± 0.04[c]	0.91±0.08
Cl-IBMECA	> 5.3	6.19 ± 0.04	> 5.3	8.89 ± 0.05 6.80 ± 0.04	9.04 ± 0.04[d] 7.06 ± 0.03	0.63±0.05
DPCPX	8.80 ± 0.03	6.58 ± 0.04	7.40 ± 0.04	5.94 ± 0.06	8.65 ± 0.06[a]	1.14±0.10
SCH 58261	6.26 ± 0.04	8.60 ± 0.05	> 5.3	> 5.3	8.56 ± 0.03[b]	1.06±0.09
MRE 2029F20	6.55 ± 0.03	> 5.3	8.46 ± 0.05	> 5.3	8.57 ± 0.06[c]	1.11±0.11
MRE 3008F20	5.93 ± 0.04	6.72 ± 0.03	5.83 ± 0.03	9.05 ± 0.05	8.55 ± 0.04[d]	0.99±0.08

Affinity values are represented as pK_H, pK_L and pKi values; n_H = Hill coefficient.

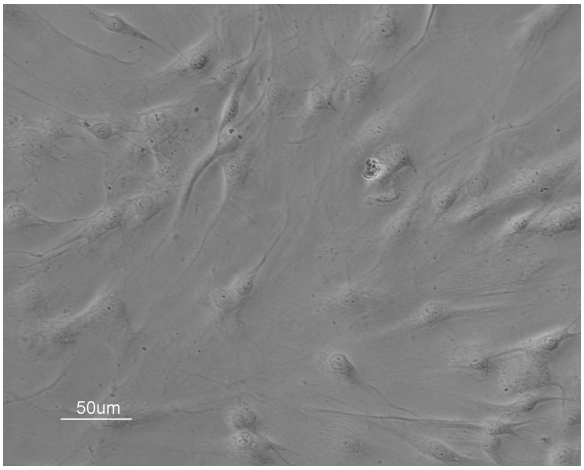
Table 3.2: Modulation of Gi, PI3K and Gs pathways in different inflammatory responses on human fibroblast-like synoviocytes.

	LPS (10 µg/m^{1})	*Cl-IB-MECA (1 µg/m^{1})*	*pertussis toxin (100 ng/m1) Cl-IB-MECA (1 µg/ml)*	*LY294002 (25 µg/ml) Cl-IB-MECA (1 µg/ml)*	*Forskolin (1 µg/ml)*
P-p38	172 ± 10	135 ± 6 [a]	133 ± 7	175 ± 8 [c]	128 ± 8 [b]
NF-kB (p50)	169 ± 14	110 ± 12 [b]	104 ± 10	173 ± 13 [d]	109 ± 9 [b]
NF-kB (p65)	353 ± 14	234 ± 12 [b]	242 ± 13	338 ± 14 [d]	226 ± 11 [b]
TNF-α	358 ± 21	128 ± 13 [b]	134 ± 12	344 ± 19 [d]	136 ± 14 [b]
IL-8	455 ± 20	262 ± 11 [b]	278 ± 15	437 ± 22 [d]	287 ± 14 [b]

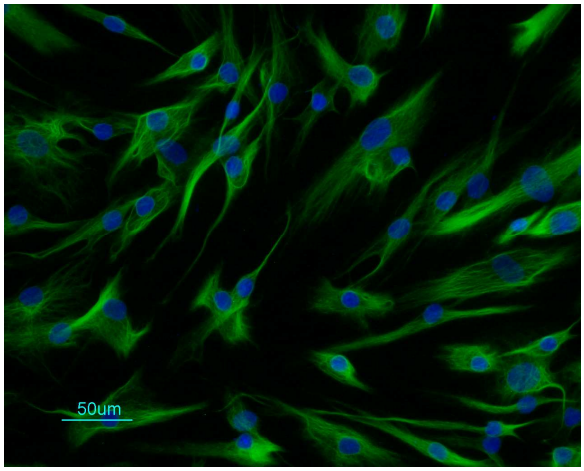
The data are expressed as % of control ± SEM. [a] $p<0.02$ versus LPS; [b] $p<0.01$ versus LPS; [c] $p<0.02$ versus Cl-IB-MECA; [d] $p<0.01$ versus Cl-IB-MECA.

Figure 3.1: Culture of human fibroblast-like synoviocytes. In A, phase contrast and in B vimentin expression by immunofluorescence. Nuclei were counterstained in blue with DAPI. Original magnification, ×200. In C CD14 and vWF mRNA expression in macrophages (MC), endothelial cells (EC) and in fibroblast-like synoviocytes (S).

A



B



C

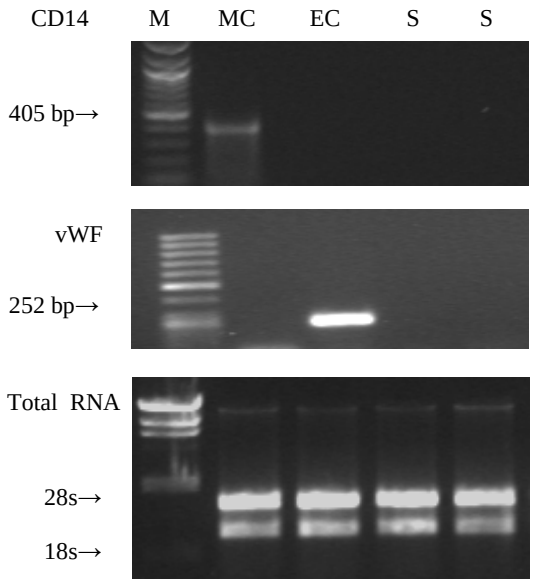


Figure 3.2: mRNA expression of ARs (A) and representative western blotting analysis (B) in human fibroblast-like synoviocytes. Densitometric analysis for ARs were also shown (C).

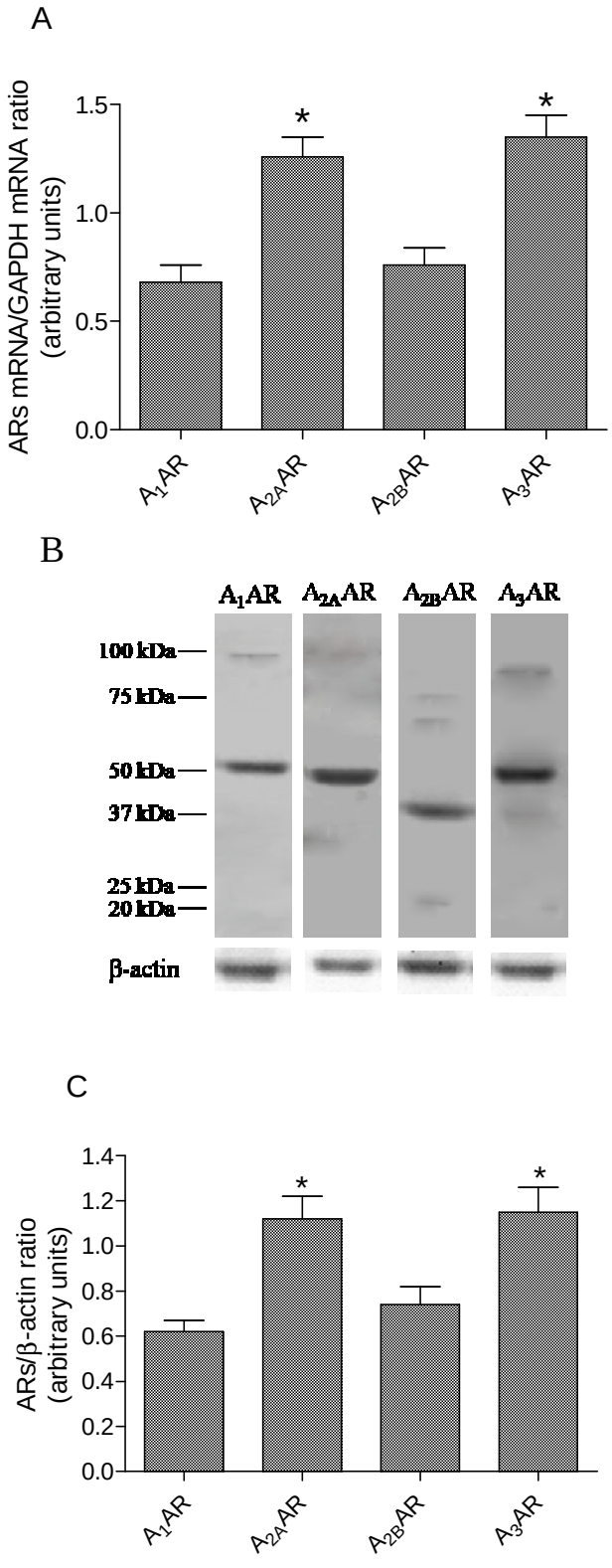


Figure 3.3: Saturation curves (A) and Scatchard plot (B) of [^{3}H]DPCPX, [^{3}H]ZM 241385, [^{3}H]MRE 2029F20, [^{3}H]MRE 3008F20 binding to A_1, A_{2A}, A_{2B} and A_3 ARs in human fibroblast-like synoviocytes, respectively.

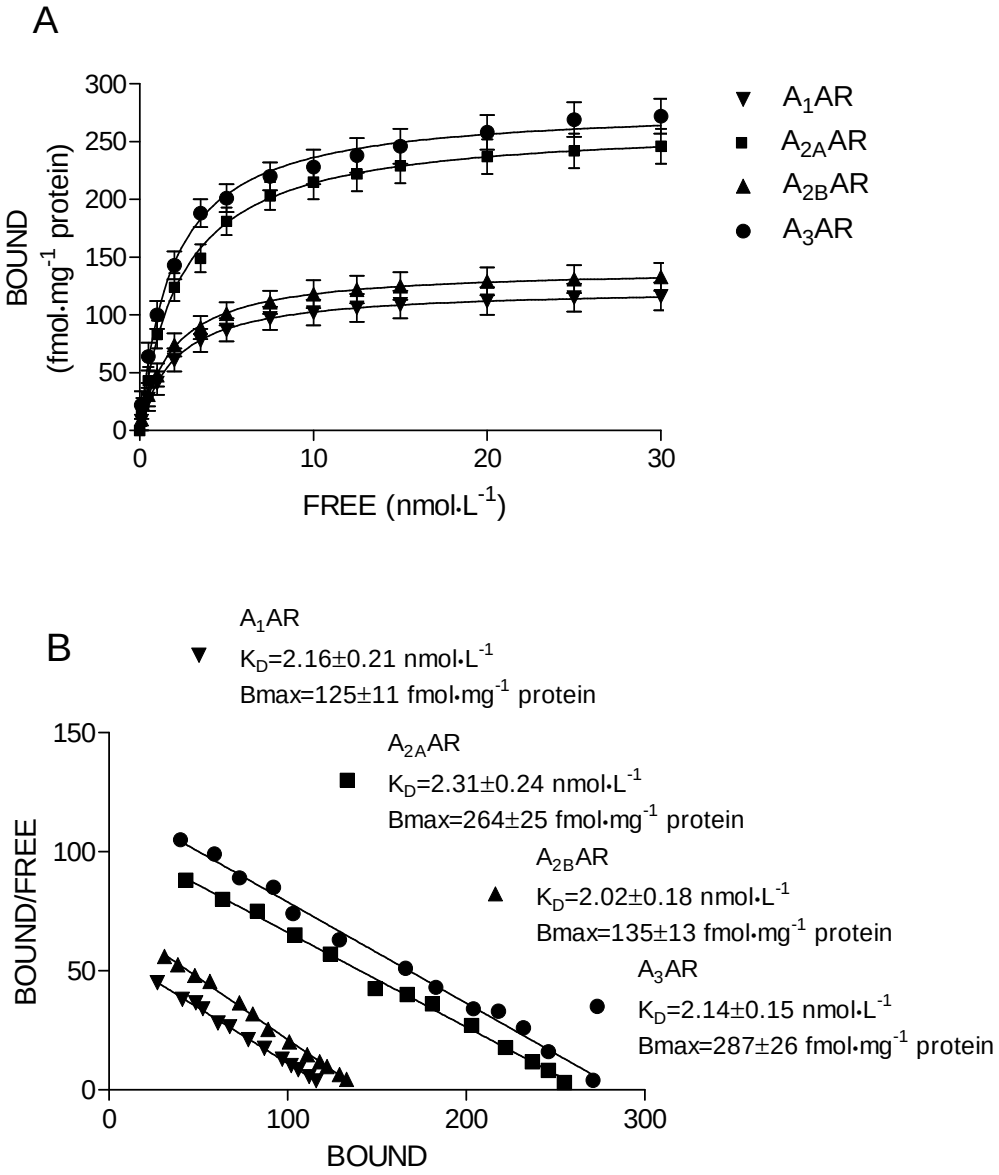


Figure 3.4: Affinity values of selected A_1, A_{2A}, A_{2B} and A_3 adenosine agonists and antagonists obtained by using competition binding experiments versus A_1 (A), A_{2A} (B), A_{2B} (C) and A_3 (D) ARs.

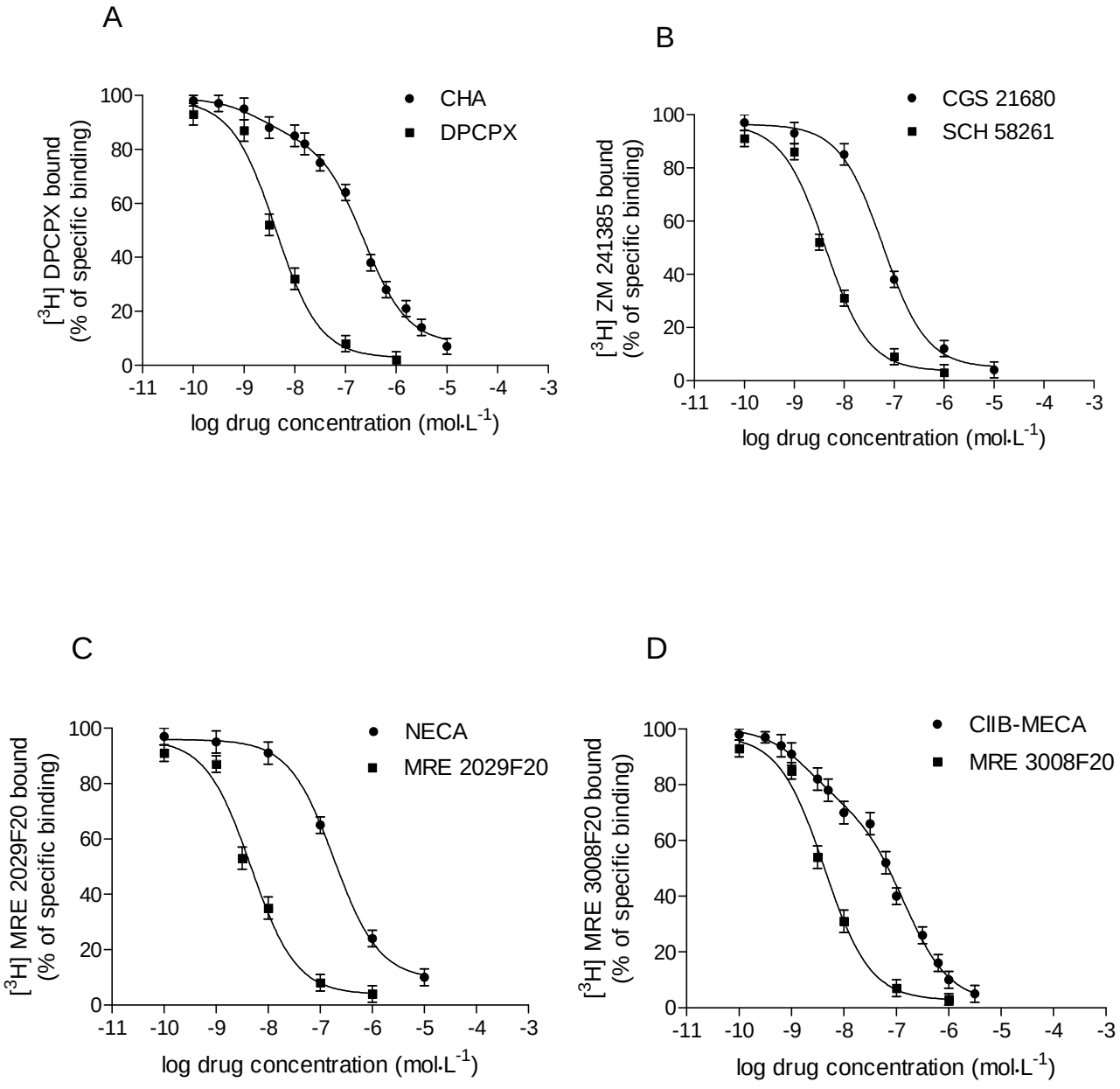
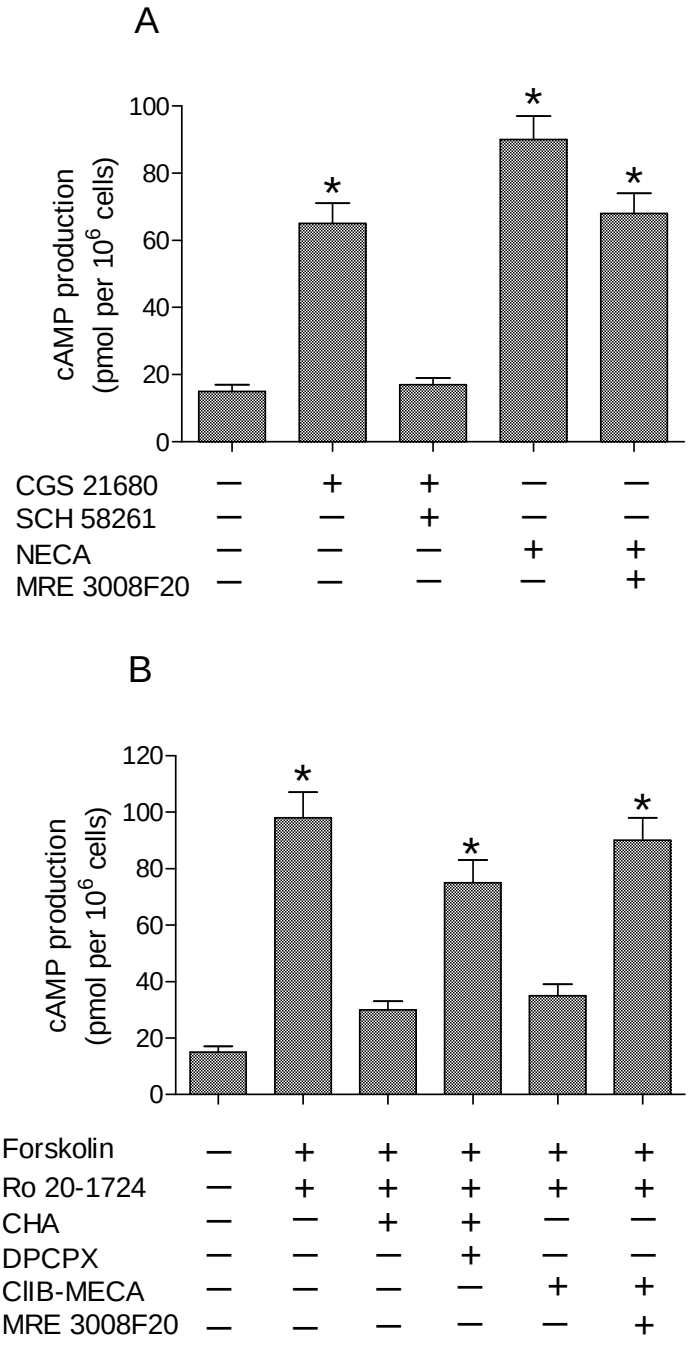
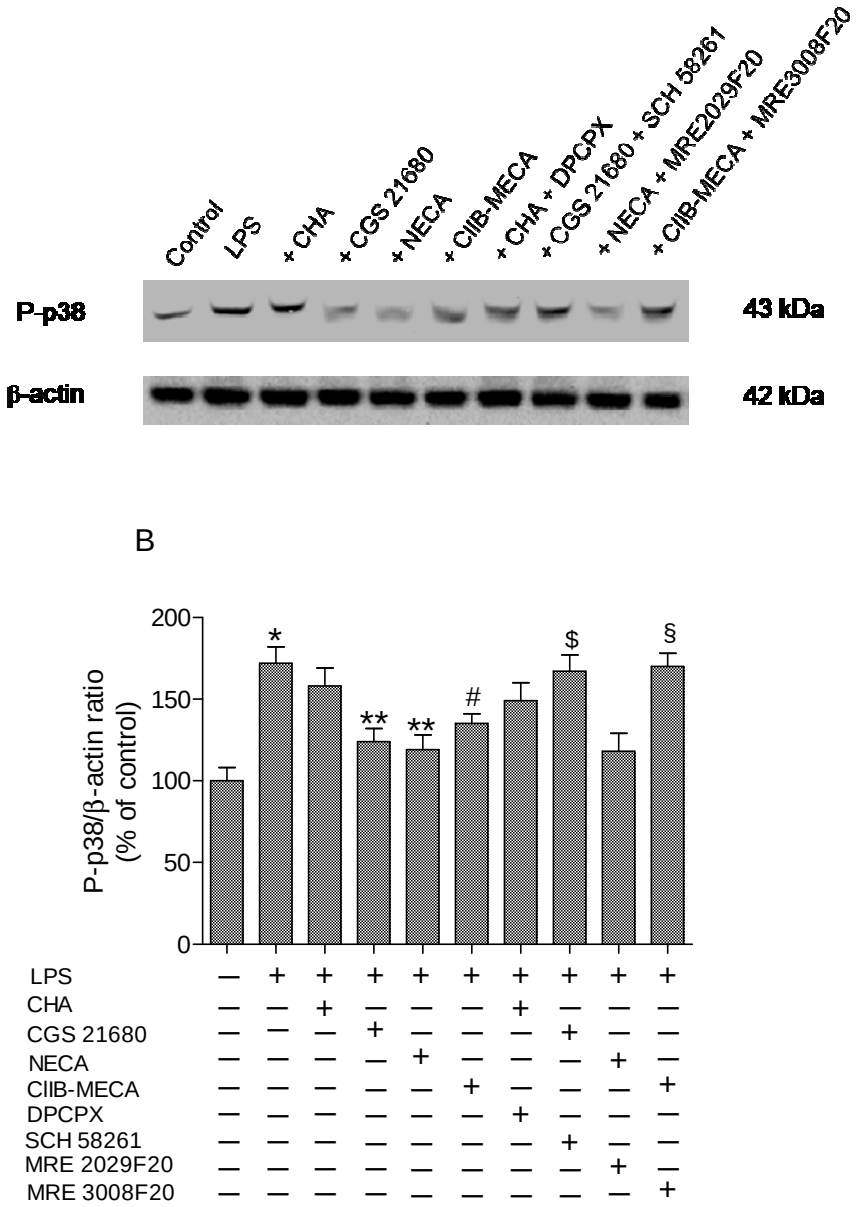


Figure 3.5: Stimulatory effect on cAMP levels of CGS 21680 and NECA in the absence and in the presence of SCH 58261 and MRE 2029F20, respectively (A). Inhibitory effect of CHA and Cl-IB-MECA in the absence and in the presence of DPCPX and MRE 3008F20, respectively (B).



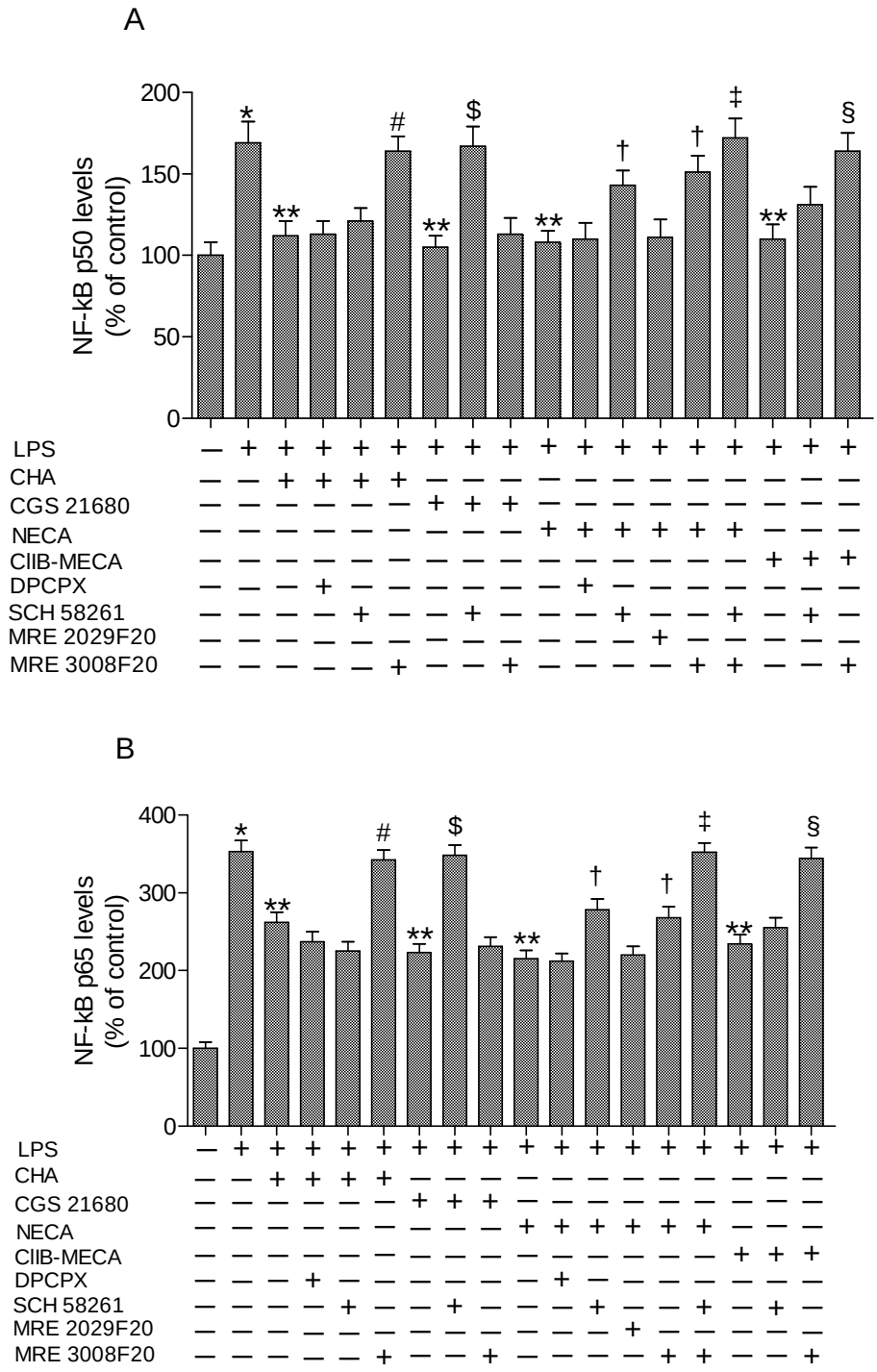
* $p<0.01$ versus control conditions.

Figure 3.6: Western blotting analysis of P-p38 in the absence and in the presence of LPS. The effect of examined adenosine agonists and antagonists was also evaluated (A). Densitometric analysis of the bands obtained were also shown (B).



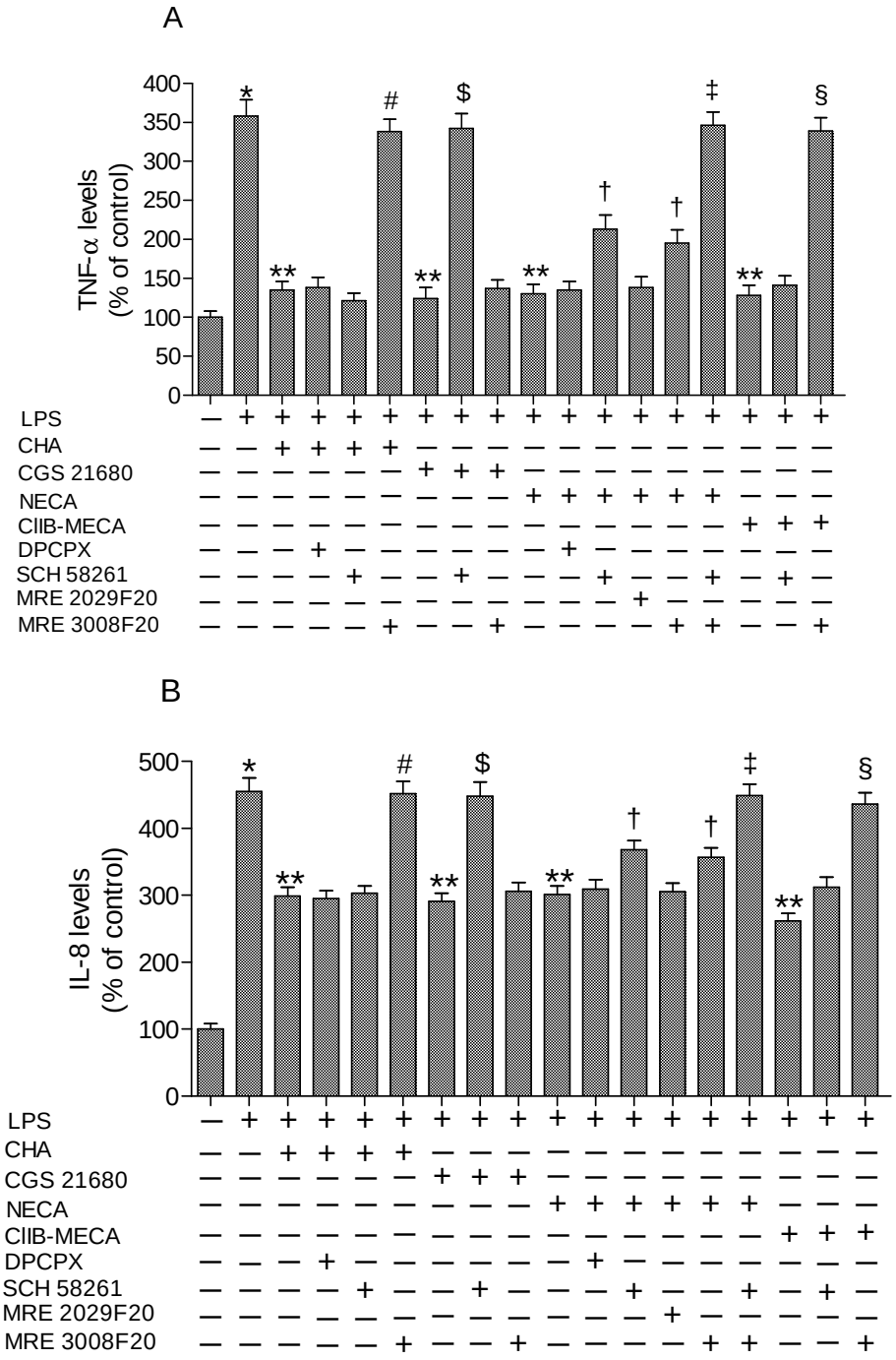
*, p< 0.01 versus control conditions; **, p< 0.01 versus LPS conditions; #, p< 0.02 versus LPS conditions; $, p< 0.01 versus CGS 21680; §, p< 0.02 versus Cl-IB-MECA.

Figure 3.7: Effect of adenosine agonists and antagonists in human fibroblast-like synoviocytes on NF-kB activation which was evaluated by detecting phosphorylated p50 (A) and p65 (B) proteins in nuclear extracts.



*, p<0.01 versus control conditions. **, p<0.01 versus LPS conditions; #, p< 0.01 versus CHA; $, p< 0.01 versus CGS 21680; †, p< 0.05 versus NECA; ‡, p< 0.01 versus NECA; §, p< 0.01 versus Cl-IB-MECA.

Figure 3.8: TNF-α (A) and IL-8 (B) levels in human synoviocytes in control conditions and stimulated by LPS (10 µg/ml). TNF-α and IL-8 levels were also calculated in the presence of adenosine agonists and antagonists.



*, p<0.01 versus control conditions. **, p<0.01 versus LPS conditions; #, p< 0.01 versus CHA; $, p< 0.01 versus CGS 21680; †, p< 0.05 versus NECA; ‡, p< 0.01 versus NECA; §, p< 0.01 versus Cl-IB-MECA.

DISCUSSION

Chronic inflammatory processes are based on a sustained and tightly regulated communication network among different cells types. It is generally accepted that fibroblast-like synoviocytes have a key function in the development of sustained inflammation in joint diseases such as OA. The aetiology of OA is multifactorial and includes the release of both systemic and local biochemical factors (Peat et al., 2001). Recently, growing evidence suggest that synovial inflammation have an important role in the pathophysiology of OA contributing to signs and symptoms of the disease (Liu et al., 2009; Sutton et al., 2009). The activation by inflammatory stimuli of fibroblast-like synoviocytes mediate the production of different chemokines, cytokines and matrix metalloproteinases (Georganas et al., 2000; Nanki et al., 2001). There is growing evidence that pro-inflammatory mediators could play critical roles in the development of inflammation and damage in joint tissues (Wen et al., 2006; Inoue et al., 2005). Several studies have indicated that adenosine, via stimulation of its receptors, is involved in the modulation of inflammatory processes (Palmer and Trevethic, 2008; Ham and Rees, 2008; Gessi et al., 2008). In particular, A_{2A} AR agonists inhibit cartilage damage when used in the treatment of septic arthritis by diminishing IL-8 expression and reduce rat adjiuvant induced arthritis (Cohen et al., 2005). In fibroblast-like synoviocytes the selective involvement of A_{2A} and A_3 ARs in the immunomodulatory actions of methotrexate has been studied (Montesinos et al., 2003; Cronstein, 2005). Recently, A_3 AR stimulation inhibits human fibroblast-like synoviocytes growth and the inflammatory manifestations of arthritis (Ochaion et al., 2008). The purpose of the present paper was to document the expression and the binding parameters of A_1, A_{2A}, A_{2B} and A_3 ARs in human fibroblast-like synoviocytes derived from OA patients. ARs are coexpressed in these cells and were investigated through mRNA, western blotting analysis and saturation binding experiments. To exactly quantify the affinity and density of ARs, saturation binding studies were performed. In human synoviocytes ARs affinities (K_D, nM) were in the nanomolar range and the receptor densities (Bmax, fmol/mg protein) were from 125 to 287 fmol/mg protein. No data are present in literature on the ARs binding parameters in human fibroblast-like synoviocytes. Moreover, binding and functional characterization was previously performed in

bovine fibroblast-like synoviocytes. In these cells, ARs affinity was similar in nanomolar range to those observed in human fibroblast-like synoviocytes. ARs density was higher in human than bovine fibroblast-like synoviocytes (Varani et al., 2008). The competition binding experiments in human fibroblast-like synoviocytes were performed with the aim to calculate the affinity of adenosine agonists and antagonists which have been also studied in functional assays. As expected, competition of [^{3}H] DPCPX and [^{3}H] MRE 3008F20 by increasing concentrations of CHA and Cl-IB-MECA, respectively revealed two binding sites for these agonists probably due to the presence of two different high and low receptor affinity states. On the contrary, competition binding curves with antagonists were monophasic (Varani et al., 2000; Merighi et al., 2001). In addition, competition of [^{3}H] ZM 241385 and [^{3}H] MRE 2029F20 by increasing concentrations of CGS 21680 and NECA, respectively showed simple inhibition curves excluding the involvement of multiple affinity states because of the tight coupling between A_2 ARs and Gs proteins (Varani et al., 1998; Gessi et al., 2005). Affinity values of adenosine agonists and antagonists versus human ARs expressed in CHO or HEK 293 cells were closely similar to those obtained in human fibroblast-like synoviocytes. CGS 21680 and Cl-IB-MECA were selective versus A_{2A} and A_3 ARs, CHA was able to interact with A_1 and A_3 ARs whilst NECA bound all ARs. The adenosine antagonists, DPCPX, SCH 58261, MRE 2029F20 and MRE 3008F20 chosen in this study are selective versus A_1, A_{2A}, A_{2B} and A_3 ARs, respectively. Another purpose of the present study was to investigate the ARs functional activities in human fibroblast-like synoviocytes where adenosine agonists and antagonists were able to modulate cAMP production. As expected CGS 21680 and NECA stimulated adenylyl cyclase activity whereas CHA and Cl-IB-MECA decreased cAMP production. These adenosine agonist effects have been blocked by selected antagonists with the aim to discriminate selectively the involvement of specific ARs. These data are in agreement with those reported in human or bovine fibroblast-like synoviocytes where A_{2A} and A_{2B} ARs are coupled positively to adenylyl cyclase whereas A_1 and A_3 ARs are linked to Gi proteins and inhibit cAMP production (Boyle et al., 1996; Varani et al., 2008). There is a large body of evidence suggesting that p38 MAPK represents one key signal transduction pathway crucial for the induction and maintenance of chronic inflammation (Westra and Limburg, 2006). This network comprises the

extracellular mediators such as cytokines, chemokines and matrix-degrading proteases which orchestrate the participation of the cells in chronic inflammatory process (Karin, 2005). The mirrors of this outside communication world are intracellular transcription factor pathways such as NF-kB, which shuttle information about inflammatory stimuli to the cell nucleus (Pomerantz and Baltimore, 2002). To address this issue, p38 MAPK activation was studied following ARs modulation in human fibroblast-like synoviocytes. We found that the stimulation of A_{2A} and A_3 ARs by using CGS 21680 and Cl-IB-MECA, respectively mediated a significant decrease of the phosphorylated, hence activated form of p38 MAPK. In literature are present few papers on human fibroblast-like synoviocytes and functional response of ARs (Boyle et al., 1996; Ochaion et al., 2008). On the other hand these data are in agreement with those previously reported in human pro-monocytic U937 cells where CGS 21680 decreased phospho-p38 protein levels (Fotheringham et al., 2004). It is well known that phospho-p38 acts as a kinase implicated in the phosphorylation of the NF-kB inhibitors IkB, allowing the p50 and p65 subunits to enter the nucleus and promoting the transcription of inflammatory genes. Therefore, we have investigated the effects of adenosine agonists and antagonists on p50 and p65 subunit levels in the nuclear extract of human fibroblast-like synoviocytes. Our data demonstrated that adenosine agonists were able to reduce p50 and p65 levels primarily through the involvement of A_{2A} and A_3 ARs as revealed by the use of adenosine antagonists. In particular, the effect of CHA, that bind A_1 and A_3 ARs with high affinity and A_{2A} ARs with low affinity (Table 3.1), was reverted only by the A_3 antagonist MRE3008F20 and not by the A_1 antagonist DPCPX excluding the involvement of A_1 ARs. The effect of the A_{2A} agonist CGS 21680 was reverted by the selective A_{2A} antagonist SCH 58261, confirming the involvement of A_{2A} AR in the reduction in p50 and p65 levels. The inhibitory effect of the adenosine agonist NECA was only abrogated by SCH 58261 and MRE 3008F20, but not by the A_1 antagonist DPCPX or the A_{2B} antagonist MRE 2029F20, demonstrating the involvement of A_{2A} and A_3 ARs but not of A_1 or A_{2B} ARs. The direct role of A_3 AR in the down-regulation of nuclear NF-kB levels was further confirmed by the inhibitory effect of the A_3 agonist Cl-IB-MECA that was completely abrogated by the A_3 antagonist MRE 3008F20.

The present data strongly support several pieces of evidence performed in different cellular models such as murine microglial cells, mouse macrophages and leukemic cell line indicating that adenosine and in particular A_{2A} and A_3 stimulation mediated the inhibition of NF-kB (Lee et al., 2006; Martin et al., 2006; Majumdar and Aggarwal, 2003). The transcription factor NF-kB represents a major component of the TNF-α gene activation machinery and its activation is necessary for TNF-α production. In our study we found that the suppression of TNF-α release was mediated by A_{2A} and A_3 ARs. Our data are in agreement with those previously reported by several authors showing that A_{2A} and A_3 ARs mediated a reduction of TNF-α production (Haskò et al., 1996; Szabò et al., 1998; Haskò et al., 2000; Mabley et al., 2003). This reduction of TNF-α levels could be explained as a direct inhibition of NF-kB, thus at a transcriptional level (Lee et al., 2006). Alternatively, it is also possible that A_{2A} and A_3 ARs-mediated inhibition of TNF-α production is closely associated with p38 MAPK that has been proposed to be a key regulator of TNF-α mRNA stability and protein translation (Fotheringham et al., 2004). The cell signalling pathways initiated by pro-inflammatory events converge on the activation of p38 and NF-kB which are also implicated in the regulation of IL-8 expression, a critical mediator of tissue inflammation. As a result of p38 and NF-kB inhibition, A_{2A} and A_3 ARs stimulation resulted in a decrease of IL-8 production. Previous contrasting studies obtained in different cells have reported that adenosine acts as a positive or negative regulator of IL-8 suggesting that this disparity of the effect could be in part explained by the presence of different target cells or tissues (Jijon et al., 2005; Murakami et al., 2001). To better investigate the role of G proteins in A_{2A} or A_3 ARs functionality, selective experiments were performed with the aim to evaluate the effect of Cl-IB-MECA in the presence of Gi protein or PI3K inhibitors. We found that the A_3-mediated reduction in NF-kB activation and cytokines release was not affected by the block of Gi proteins but only by the inhibition of PI3K suggesting that, for these responses, A_3 AR signals through a PI3K pathway. In contrast, the A_{2A} AR-mediated reduction of inflammatory responses was most likely due to the activation of Gs protein and to the increase in cAMP levels, as demonstrated by the use of adenylyl cyclase direct activator forskolin that leads to similar responses to those of A_{2A} ARs. Clearly, further studies will be needed regarding the differential roles of other MAPKs and transcription factors on

various inflammatory mediators to delineate the mechanisms underlying cytokine production by adenosine signalling. In conclusion, the novel findings of this study in human fibroblast-like synoviocytes from OA patients are represented by the presence of ARs that are also quantified by high levels of density. The functional results revealed the direct involvement of A_{2A} and A_3 ARs in the inhibition of inflammatory cascade in human fibroblast-like synoviocytes. Taken together these results suggest that ARs could represent potential therapeutic pharmacological target in the complex pathways regulating inflammatory processes in joint diseases.

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CHAPTER 4:

Binding thermodynamic characterization of human $P2X_1$ and $P2X_3$ purinergic receptors.

INTRODUCTION

P2 purinergic receptors are divided in two subfamilies: G-protein coupled (P2Y) and ligand-gated ion channels (P2X). In mammalian cells eight P2Y ($P2Y_{1,2,4,6,11,12,13,14}$), 7 homomeric P2X ($P2X_{1-7}$) and four heteromeric ($P2X_{2/3}$, $P2X_{1/5}$, $P2X_{2/6}$, $P2X_{4/6}$) receptors have been cloned and characterized pharmacologically (North, 2002). Both of these receptor families are activated by the presence of extracellular ATP, an important local regulatory factor under physiological, inflammatory and neuropathic pain conditions (North et al., 2000; Burnstock, 2006; Sawynok, 2007). Briefly, P2X receptors are abundantly distributed, and functional responses have been reported in neurons, glia, epithelia, endothelia, bone, muscle and hemopoietic tissues (Khakh and North, 2006). The widespread expression of $P2X_1$ receptors suggests that ATP may contribute in various pathophysiological conditions (Burnstock and Knight, 2004). $P2X_3$ receptors have been found primarily localised to specific subsets of sensory nociceptor neurones, and there is now much evidence that $P2X_3$ homomeric and $P2X_{2/3}$ heteromeric receptors in sensory pathways are involved in neuropathic and chronic pain (Kennedy, 2005; Jarvis, 2003). Pharmacological methods and manipulations, that can differentiate between $P2X_1$ and $P2X_3$ receptors, could be very useful in the development of $P2X_1$ or $P2X_3$ receptor-specific ligands (Abbracchio and Williams, 2001; King and Notrh, 2000). This is especially important because the rapidly desensitising kinetics and the agonist and antagonist pharmacology of these two P2X receptors are remarkably similar to each other (North, 2000; North, 2002; Burnstock, 2006). ATP, and the ATP analogues, BzATP, 2meSATP and αβmeATP show equipotent agonist profiles at $P2X_1$ and $P2X_3$ receptors (Jacobson et al., 2002). Considerable changes in binding affinity of purinergic antagonists are also reported probably due to the little information available with respect to the regions of the receptor involved in antagonist binding (North, 2000, 2002). More recently, a submicromolar affinity, non-nucleotide antagonist, A 317491, has been described which is more than 100-fold selective for $P2X_3$/$P2X_{2/3}$ receptors over $P2X_1$ receptors in electrophysiological and calcium flux functional assays (Jarvis et al., 2004; Burgard et al., 2000). Radioligand binding techniques have had limited success in differentiating between endogenous or recombinant P2X receptors, probably due to the lack of highly

selective and potent ligands (Jacobson et al., 2002). The ATP bioisosteres, [^{3}H]αβmeATP, [^{35}S]ADPβS and [^{35}S]ATPγS have been used in ligand-binding studies of purinergic receptors in various tissues and recombinant expression systems (Jarvis et al., 2004; Bo and Burnstock, 1990; Michel et al., 1995, 1996; Schafer and Reiser, 1997). In spite of the problems associated with the use of these radioligands, information obtained from specific binding assays has long proved invaluable for screening potential drug candidates, for basic pharmacological characterization of receptor subtypes and for identification of signal transduction pathways. It can be of interest to obtain determinations of drug-receptor binding association (K_A) and dissociation ($1/K_A$ or K_D) constants over a range of temperatures, in contrast to the single-point temperature assays, which add significant information on the molecular mechanisms involved in the drug-receptor interaction (Borea et al., 2000). Determination of K_A or K_D values makes it possible to calculate the standard free energy $\Delta G° = -RTlnK_A = RTlnK_D$ (T=298.15 K), but not its two components, the equilibrium standard enthalpy ($\Delta H°$) and entropy ($\Delta S°$) as defined by the Gibbs equation $\Delta G° = \Delta H° - T\Delta S°$. As a consequence, van't Hoff plot analysis was performed to obtain K_D values over a range of temperatures and to obtain the thermodynamic terms of this equation. From such analysis, it is generally proposed that standard enthalpy is a quantitative indicator of the changes in intermolecular bond energies, such as hydrogen bonding and van der Waals interactions, occurring during the binding. In addition standard entropy can be considered an indicator of the rearrangements undergone by the solvent, normally water molecules, during the same process (Borea et al., 2000). In the last few years it has been shown that the $\Delta H°$ and $\Delta S°$ values of drug interaction with a defined receptor can often give a simple in vitro way to discriminate agonists from antagonists suggesting the manner in which the drug interferes with the signal transduction pathways. Such "thermodynamic discrimination" reveals that the binding of agonists may be entropy-driven and that the antagonists are enthalpy-driven, or vice versa. At the present fourteen receptor systems have been extensively studied so far from thermodynamic point of view: eleven of these show the agonist-antagonist discrimination and three are not discriminated (Borea et al., 1996, 1998, 2000; Merighi et al., 2002; Gilli et al., 2005). In particular, all the ligand-gated ion channel receptors, i.e. glycine, GABA, $5HT_3$ and neuronal nicotinic receptors have been reported to discriminate

in vitro the effect of their agonists and antagonists (Maksai, 2001; Gessi et al., 1999; Borea et al., 2004). Analysis of thermodynamic data of drug-receptor interactions appears to be an effective tool in the study of the role, at the molecular level, played during the binding of the ligands (Stoop et al., 1997; Froldi et al., 1997; Borea et al., 1995). From this background, the aim of this study was to determine the thermodynamic parameters of typical agonists and antagonists in HEK 293 cells transfected with human $P2X_1$ and $P2X_3$ receptors by using [^{3}H]αβmeATP radioligand binding assays and van't Hoff analysis in order to gain further insight into possible differences in pharmacological properties between these two therapeutically important P2X receptor subtypes. This research could provide information in the development of novel and potent $P2X_1$ and $P2X_3$ purinergic ligands

MATERIALS AND METHODS

Materials

[^{3}H]αβmeATP ([^{3}H]αβmethyleneATP specific activity 15.0 Ci mmol^{-1}) was obtained from NEN-Perkin Elmer Life and Analytical Sciences (USA). ATP, ADP, αβmeATP, BzATP (benzoilATP), TNP-ATP (2'-3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate), suramin, NF023 (8,8'-[carbonylbis (imino-3,1-phenylene carbonylimino)]bis(1,3,5-naphthalene-trisulfonic acid), PPADS (pyridoxal 5-phosphate 6-azophenyl-2',4'-disulphonic acid), A317491 (5-({[3-phenoxybenzyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino}carbonyl)-1,2,4-benzenetri-carboxylic acid) and apyrase were obtained from Sigma-Aldrich Advanced Sciences (Milan, Italy). NF279 (8,8'-[carbonylbis(imino-4,1-phenylenecarbonyl-imino-4,1-phenylene carbonyl- imino)] bis-1,3,5-naphthalene-trisulphonic acid)and 2meSATP (2methylSATP) were obtained from Tocris Cookson Ltd (Bristol, 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 kindly provided by Prof. A. Surprenant (Institute of Molecular Physiology, University of Sheffield, Sheffield, England, UK). Methods of maintenance of HEK293 cells and their stable

transfection with $hP2X_1$ and $hP2X_3$ receptors cDNA have been described previously (Stoop et al., 1997). Briefly, the cells were grown adherently and maintained in DMEM/F12, containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and Geneticin (G418, 0.2 mg/ml) at 37°C in 5% CO_2/95% air. Cells were split 2 or 3 times weekly at a ratio of 1:5. For membrane preparation, the culture medium was removed and the cell suspension was washed with PBS and scraped in ice-cold hypotonic buffer. The cell suspension was homogenized with Polytron (Kinematica, Switzerland) at a setting of 6 for 40 sec and the homogenate was spun for 30 min at 100,000 *g* and frozen at -80°C until binding experiments.

Effect of various factors on purinergic receptor binding assays

The effect of pH at the different values (6.0, 7.0, 7.4, 8.0, 9.0) on the [^{3}H]αβmeATP binding at the 3 nM concentration was evaluated and obtained incubating in Tris HCl 50 mM the HEK-293 membranes containing $hP2X_1$ or $hP2X_3$ purinergic receptors. The effect of different concentrations of Ca^{2+} (from 0 to 10 mM) and Mg^{2+} (from 0 to 10 mM) was also investigated by using 3 nM of [^{3}H]αβmeATP as radioligand in Tris HCl 50 mM pH 7.4 and HEK-293 membranes containing $hP2X_1$ or $hP2X_3$ purinergic receptors. The effect of Apyrase (1 U/ml for 30 min at 37°C) was evaluated incubating the $hP2X_1$ or $hP2X_3$ membranes in Tris HCl 50 mM pH 7.4 $CaCl_2$, 4 mM and 3 nM of [^{3}H]αβmeATP as radioligand. Non specific binding was determined in the presence of 10 μM αβmeATP (Froldi et al., 1997). After the incubation time (40 min at 5°C) bound and free radioactivity were separated by rapid filtration through Whatman GF/B glass-fiber filters using a Brandel instrument. The filter bound radioactivity was counted on a Scintillation Counter Tri Carb Packard 2500 TR (efficiency 57%).

Kinetics of [^{3}H]αβmeATP binding

Kinetic studies of 3 nM [^{3}H]αβmeATP were performed by incubating membranes in Tris HCl 50 mM, $CaCl_2$ 4 mM pH 7.4 obtained as described above in a thermostatic bath at 5°C. For the measurement of the association rate of human $P2X_1$ receptors, the reaction was terminated at different times (from 1 to 80 min) by rapid filtration under vacuum, followed by washing four times with ice

cold buffer. Similarly, for human $P2X_3$ receptors the reaction was terminated from 10 sec to 30 min by rapid filtration. For the measurement of the dissociation rate, the samples were incubated at 5°C for 40 min and then 10 μM αβmeATP was added to the mixture. The reaction was terminated for $P2X_1$ receptors from 1 to 40 min and for $P2X_3$ receptors from 30 sec to 40 min.

Saturation and competition binding experiments

Saturation and competition binding assays were performed on HEK293-h$P2X_1$ and HEK293-h$P2X_3$ membranes at 5, 10, 15, 20, 25 and 30°C, in a thermostatic bath assuring a temperature of ± 0.1°C. Analogous experiments in HEK293 wild type and in HEK293 transfected with the vector alone were executed. Saturation binding experiments of [^{3}H]αβmeATP (0.1 to 50 nM) to the membranes previously obtained were performed in Tris-HCl 50 mM, $CaCl_2$ 4 mM pH 7.4 for an incubation time ranged from 40 min at 5°C to 90 min at 30°C according to the results of previous time-course experiments. Competition experiments of 3 nM [^{3}H]αβmeATP were performed in the same buffer described above and at least 8-10 different concentrations of $P2X_1$ and $P2X_3$ agonists or antagonists studied. Non specific binding was determined in the presence of 10 μM αβmeATP (Froldi et al., 1997). Bound and free radioactivity were separated by rapid filtration through Whatman GF/B glass-fiber filters using a Brandel instrument. The filter bound radioactivity was counted on a Scintillation Counter Tri Carb Packard 2500 TR (efficiency 57%). The affinity values expressed as K_D or Ki were used in the thermodynamic parameter determination.

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: $\Delta G° = \Delta H° - T\Delta S°$. The standard free energy was calculated as $\Delta G° = -RT\ln K_A$ at 298.15 K, the standard enthalpy, $\Delta H°$, from the van't Hoff plot $\ln K_A$ versus (1/T) (the slope of which is $-\Delta H°/R$) and the standard entropy as $\Delta S° = (\Delta H° - \Delta G°)/T$ with T = 298.15 K and R = 8.314 J/K/mol (Borea et al., 1995, 2000, 2004).

Data analysis

Kinetic, saturation and competition binding experiments were analysed with the program Ligand (Bradford, 1976) which performs weighted non linear least-squares curve fitting program. The protein concentration was determined according to a Bio-Rad method with bovine albumin as reference standard (Munson and Rodbard, 1980). All experimental data are reported as mean ± SEM of three or four independent experiments performed in duplicate.

RESULTS

Effect of various factors on purinergic receptor binding assays

Preliminary assays were performed to determine the experimental conditions for the evaluation of specific [^{3}H]αβmeATP binding. The incubation of the HEK293-hP2X$_1$ and HEK293-hP2X$_3$ membranes with apyrase (1U/ml) with the aim to eliminate extracellular ATP and minimize receptor desensitization during binding experiments produced no differences in the percentage of specific binding (HEK293-hP2X$_1$ = 85±8% and 88±9% or HEK293-hP2X$_3$ = 89±9% and 92±10%, respectively). The effect of pH values (from 6 to 9) on the [^{3}H]αβmeATP binding reveals that the optimal pH value for the binding experiments was around 7.4 in both cell lines examined (Figure 4.1A,C). The specific binding of [^{3}H]αβmeATP to HEK293-hP2X$_1$ (Figure 4.1B) and HEK293-hP2X$_3$ (Figure 4.1D) membranes increased of 8 and 6 fold, respectively with the raise of the Ca^{2+} concentration from 1 µM to 1 mM. On the contrary, further increasing in the calcium concentration from 1 to 10 mM led to a decline of specific [^{3}H]αβmeATP binding. The addition of Mg^{2+} (from 1 to 10 mM) in the same experimental conditions resulted in a strong reduction (80%, n=3) of the binding suggesting that the [^{3}H]αβmeATP binding was regulated in an opposite way by physiological concentrations of Ca^{2+} and Mg^{2+}, respectively.

Kinetic binding assays to human P2X$_1$ and P2X$_3$ purinergic receptors

Kinetic behaviour of [^{3}H]αβmeATP binding was studied at 5°C in HEK293-hP2X$_1$ (Figure 4.2) and HEK293-hP2X$_3$ (Figure 4.3) membranes. Figures 4.2A and 4.3A show that [^{3}H]αβmeATP binding reached equilibrium after

approximately 20 and 5 min, respectively and was stable for at least 90 min. [^{3}H]αβmeATP binding was rapidly reversed by the addition of 10 μM αβmeATP as shown in figures 4.2B and 4.3B. Association and dissociation curves in HEK293-hP2X$_1$ and HEK293-hP2X$_3$ membranes were fitted to a one component model significantly better than to a two component model ($p<0.05$). The rate constants were: k_{obs} = 0.106 ± 0.012 1/min and = 0.712 ± 0.010 1/min, respectively. The k_{+1} values were = 0.017 ± 0.002 1/min nM and = 0.133 ± 0.014 1/min nM , respectively. The apparent equilibrium dissociation constant (K_D) was estimated to be 3.29 nM and 2.35 nM, respectively.

Saturation binding assays to human P2X$_1$ and P2X$_3$ purinergic receptors

Saturation binding experiments in HEK 293 cells were performed to better characterize human P2X$_1$ and P2X$_3$ purinergic receptors and evaluate affinity (K_D) and receptor density (Bmax) values (Table 4.1). These binding parameters were determined at various temperatures by using [^{3}H]αβmeATP as radioligand at different concentrations. In both purinergic receptors examined the K_D values change with temperature and Bmax values appear to be largerly independent of it. Figures 4.4 and 4.5 illustrate saturation binding curves and Scatchard plot relative to human P2X$_1$ and P2X$_3$ purinergic receptors, respectively. Scatchard plots were linear at all temperatures investigated and computer analysis of the data failed to show a significantly better fit to a two site than to a one site binding model, indicating that only one class of high affinity binding site was present under our experimental conditions. Saturation binding experiments performed employing HEK 293 wild type and HEK 293 transfected with the vector alone failed to show significant values of specific binding (n=4 experiments, % of specific binding = 2 ± 1% and 3 ± 1%, respectively) suggesting that the P2X$_1$ and P2X$_3$ transfection is essential to the presence of specific binding.

Competition binding assays to human P2X$_1$ and P2X$_3$ purinergic receptors

Figures 4.6 and 4.7 show the dose response curves of [^{3}H]αβmeATP binding in HEK293-hP2X$_1$ and HEK293-hP2X$_3$ membranes by using typical agonists and antagonists. The order of potency in [^{3}H]αβmeATP displacement assays for purinergic agonists in HEK293-hP2X$_1$ was as follows: αβmeATP > BzATP> 2meSATP > ATP > ADP. A similar order of potency was also obtained in

HEK293-hP2X$_3$ even if the affinity values were much higher than in HEK293-hP2X$_1$ membranes. The order of potency of antagonists in HEK293-hP2X$_1$ was similar to those obtained in HEK293-hP2X$_3$ as follows: TNP-ATP > A 317491 > NF 279 > NF 023 > Suramin > PPADS and TNP-ATP > A 317491 > NF 023 > Suramin > NF 279 > PPADS, respectively.

Thermodynamic analysis to human P2X$_1$ and P2X$_3$ purinergic receptors

The van't Hoff plots show that the effect of temperature on the equilibrium binding association constants, K_A appears to be essentially linear in the range 4-30°C for purinergic ligands examined to human P2X$_1$ and P2X$_3$ purinergic receptors (Figures 4.8 and 4.9). Slopes of van't Hoff plots are positive for agonists and antagonists of P2X$_1$ purinergic receptors showing that the affinities decrease with the increase of the temperature (Table 4.2A).The slopes of van't Hoff plots regarding P2X$_3$ purinergic receptors are positive for agonists whose affinities decrease with the increase of the temperature and negative for antagonists whose affinities are improved by an increase in temperature (Table 4.2B). Final thermodynamic parameters calculated for the binding equilibria of the different compounds investigated are reported in Table 4.3. In P2X$_1$ purinergic receptors $\Delta G°$ values range from -46.0 to – 37.4 kJ/mol for agonists and from -30.1 to -25.5 kJ/mol for antagonists. In P2X$_3$ purinergic receptors $\Delta G°$ values range from -46.2 to -41.0 kJ/mol for agonists and from –39.8 to -29.3 kJ/mol for antagonists. Equilibrium standard enthalpy $\Delta H°$ and entropy $\Delta S°$ values show similar values for agonists and antagonists examined suggesting that P2X$_1$ purinergic receptors are not thermodynamically discriminated. The analysis of thermodynamic parameters of P2X$_3$ purinergic receptors shows that the binding for agonists is always enthalpy- and entropy-driven ($\Delta H°$ values ranging from -26 to -18 kJ/mol and $\Delta S°$ values from 59 to 68 J/K/mol) while for antagonists it is totally entropy-driven ($\Delta H°$ values ranging from 14 to 36 kJ/mol and $\Delta S°$ values from 149 to 249 J/K/mol). Therefore, agonists and antagonists at P2X$_3$ purinergic receptors are thermodynamically discriminated. Interestingly, the compound TNP-ATP, reported to be from a functional point of view as a purinergic antagonist (Burgard et al., 2000), shows a typical agonist behaviour with an affinity value and thermodynamic parameters strictly similar to those obtained for the other purinergic agonists. Figure 4.10 summarizes the results in the form $-T\Delta S°$ versus

$\Delta H°$ scatter plot (T = 298.15 K) showing that for human $P2X_1$ receptors the points are present in the region that characterises the enthalpy and entropy-driven binding without a thermodynamically discrimination. In human $P2X_3$ purinergic receptors antagonists are clustered in the endothermic region ($14 \leq \Delta H° \leq 36$ kJ/mol) with large positive entropy values ($-74.20 \leq -T\Delta S° \leq -44.40$ kJ/mol) revealing that their binding is totally entropy-driven. Agonist binding is enthalpy and entropy-driven ($-26 \leq \Delta H° \leq -18$ kJ/mol and $-21.75 \leq -T\Delta S° \leq -17.58$ kJ/mol).

Table 4.1: Binding parameters of HEK293-hP2X$_1$ and HEK293-hP2X$_3$ membranes (A) and thermodynamic parameters for the binding equilibrium of [^{3}H]αβmeATP in the same substrates (B).

(A) [^{3}H]αβme ATP binding	5°C (278 K)	10°C (283 K)	15°C (288 K)	20°C (293 K)	25°C (298 K)	30°C (303 K)
HEK293-hP2X$_1$						
K_D (nM)	3.2±0.3	4.1±0.4	4.8±0.4	6.4±0.6	8.1±0.7	11.1±1.1
Bmax (fmol/mg protein)	3120±290	3200±310	3300±320	3400±330	3050±315	3500±325
HEK293-hP2X$_3$						
K_D (nM)	2.6±0.3	3.5±0.4	4.0±0.4	4.9±0.5	6.2±0.6	7.5±0.7
Bmax (fmol/mg protein)	18800±1600	18900±1650	19000±1700	19200±1750	18500±1520	19500±1800

(B) Cell lines	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol/K)
HEK293-hP2X$_1$	-46.0±0.2	-32±3	46±3
HEK293-hP2X$_3$	-46.7±0.2	-28±2	64±6

Table 4.2A: Affinities, expressed as Ki values (nM) of selected purinergic compounds to human $P2X_1$ receptors expressed in HEK293 cells

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
Purinergic Agonists	Ki (nM)	Ki (nM)	Ki (nM)	Ki (nM)	Ki (nM)	Ki (nM)
ATP	120±10	150±14	180±15	215±20	250±24	306±28
ADP	125±11	155±13	200±16	227±18	245±22	316±30
αβmeATP	3.5±0.3	4.2±0.4	4.8±0.5	6.4±0.6	8.1±0.7	11.4±1.1
2meSATP	100±10	120±11	150±12	180±15	200±18	250±22
BzATP	60±6	90±9	113±10	137±12	168±18	200±16
Purinergic Antagonists						
Suramin	7500±650	8300±730	9600±850	11200±950	13700±1100	16700±1200
NF023	6140±500	7500±600	10000±970	11200±1050	12800±1080	14000±1270
TNP-ATP	50±4	60±5	75±7	92±8	113±10	137±15
PPADS	16700±1500	18500±1700	20400±2000	27500±2400	30400±2800	33600±3120
A317491	2500±220	3000±210	3500±250	4300±300	5000±420	5600±510
NF279	4000±300	4500±350	5000±380	5600±460	6800±610	8500±720

Table 4.2B: Affinities, expressed as Ki values (nM) of selected purinergic compounds to human $P2X_3$ receptors expressed in HEK293 cells

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
Purinergic Agonists	Ki (nM)	Ki (nM)	Ki (nM)	Ki (nM)	Ki (nM)	Ki (nM)
ATP	15±2	18±2	20±2	25±2	30±3	37±4
ADP	30±3	37±3	45±4	50±5	60±5	75±6
αβmeATP	2.9±0.3	4.1±0.4	4.6±0.5	5.3±0.5	7.6±0.6	9.2±0.8
2meSATP	5.1±0.4	6.8±0.5	8.2±0.7	9.7±0.8	11.2±1.0	13.8±1.2
BzATP	5.4±0.4	7.2±0.5	9.4±0.7	10.7±0.9	12.5±1.1	14.9±1.3
Purinergic Antagonists						
Suramin	4000±380	3730±350	3100±280	2800±245	2500±240	2100±180
NF023	3500±320	3200±300	3000±260	2700±252	2300±210	2000±195
TNP-ATP	28±3	34±3	40±4	46±4	50±5	60±5
PPADS	20000±1850	15000±1200	12000±1100	9170±840	7500±720	6000±510
A317491	250±22	226±20	185±15	137±12	100±9	70±6
NF279	10000±920	9170±810	8290±780	7500±740	6790±630	5560±450

Table 4.3: Thermodynamic parameters for the binding equilibrium of [^{3}H]αβmeATP to human $P2X_1$ (A) and $P2X_3$ (B) purinergic receptors expressed in HEK293 cells.

(A) Ligand	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/K/mol)
Purinergic Agonists			
ATP	-37.4±0.1	-25±3	43±3
ADP	-37.6±0.1	-24±2	47±3
αβmeATP	-46.0±0.2	-31±3	51±4
2meSATP	-37.8±0.1	-24±1	46±3
BzATP	-38.4±0.1	-23±2	50±4
Purinergic Antagonists			
Suramin	-27.8±0.1	-21±2	21±3
NF023	-27.6±0.1	-22±2	20±2
TNP-ATP	-39.3±0.2	-27±3	41±4
PPADS	-25.5±0.1	-20±2	17±1
A317491	-30.1±0.2	-22±3	27±2
NF279	-29.3±0.1	-19±1	34±2

(B) Ligand	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/K/mol)
Purinergic Agonists			
ATP	-42.7±0.1	-24±1	62±5
ADP	-41.0±0.1	-24±2	59±4
αβmeATP	-46.2±0.2	-26±3	68±5
2meSATP	-45.1±0.2	-26±2	65±5
BzATP	-44.9±0.1	-18±2	64±4
Purinergic Antagonists			
Suramin	-31.9±0.1	18±2	168±8
NF023	-32.0±0.1	14±1	153±8
TNP-ATP	-41.4±0.2	-20±2	73±6
PPADS	-29.3±0.1	36±3	218±9
A317491	-39.8±0.2	35±3	249±9
NF279	-29.5±0.1	15±2	149±7

Figure 4.1: Effect of pH on [^{3}H]αβmeATP binding in HEK293-hP2X$_1$ and HEK293-hP2X$_3$ membranes (A,C). Effect of Ca^{2+} on [^{3}H]αβmeATP binding in HEK293-hP2X$_1$ and HEK293-hP2X$_3$ membranes (B,D).

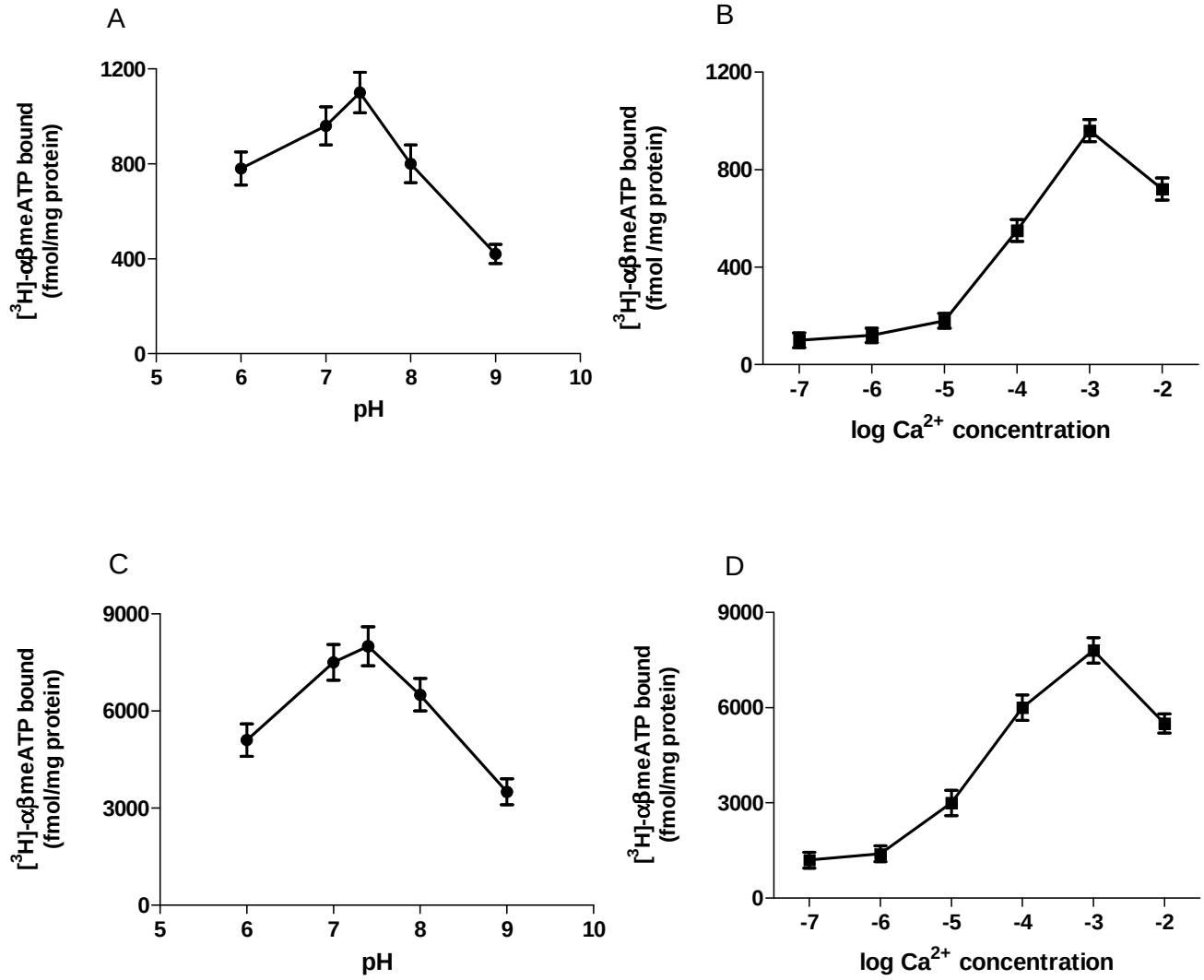


Figure 4.2: Kinetics of [^{3}H]αβmeATP binding to HEK-293-hP2X$_1$ membranes with associaton (A) and dissociation (B) curves.

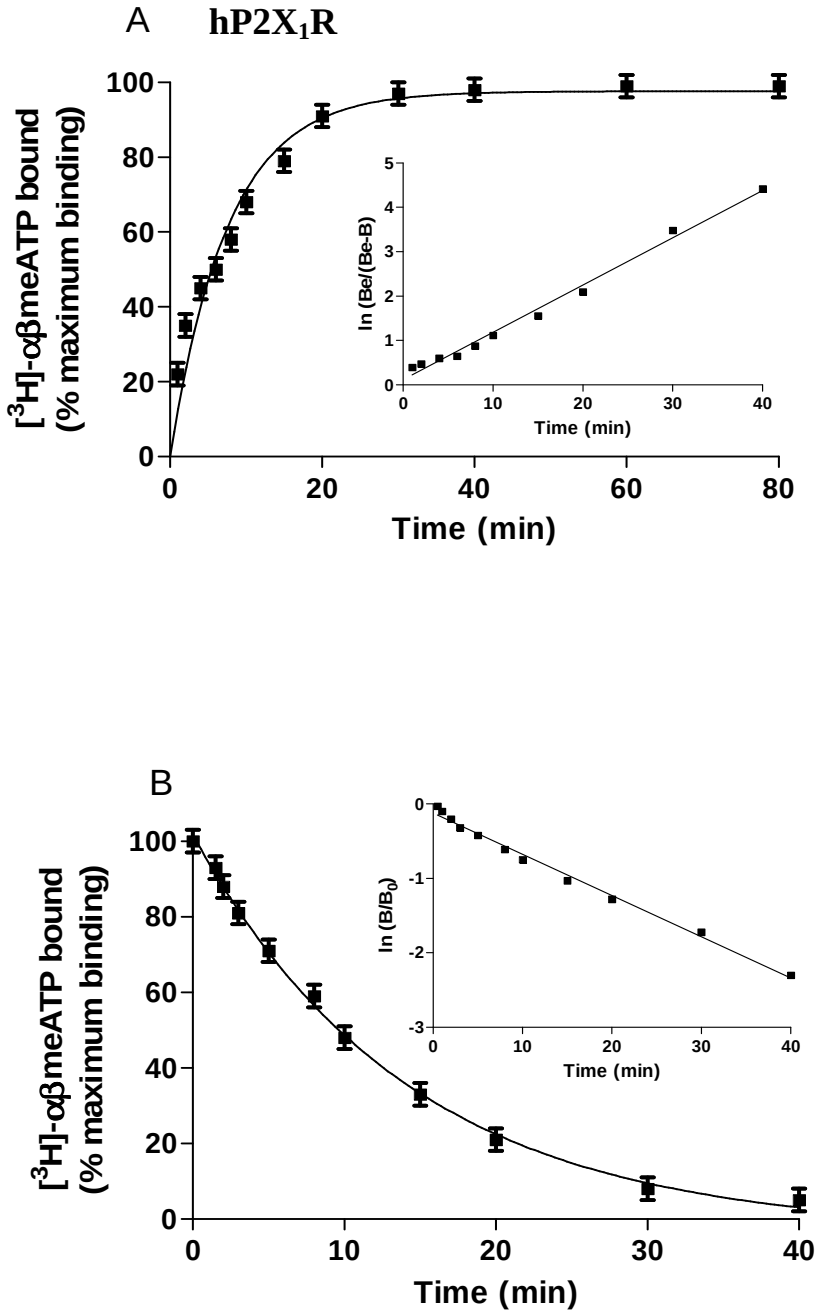


Figure 4.3: Kinetics of [^{3}H]αβmeATP binding to HEK-293-hP2X$_3$ membranes with associaton (A) and dissociation (B) curves.

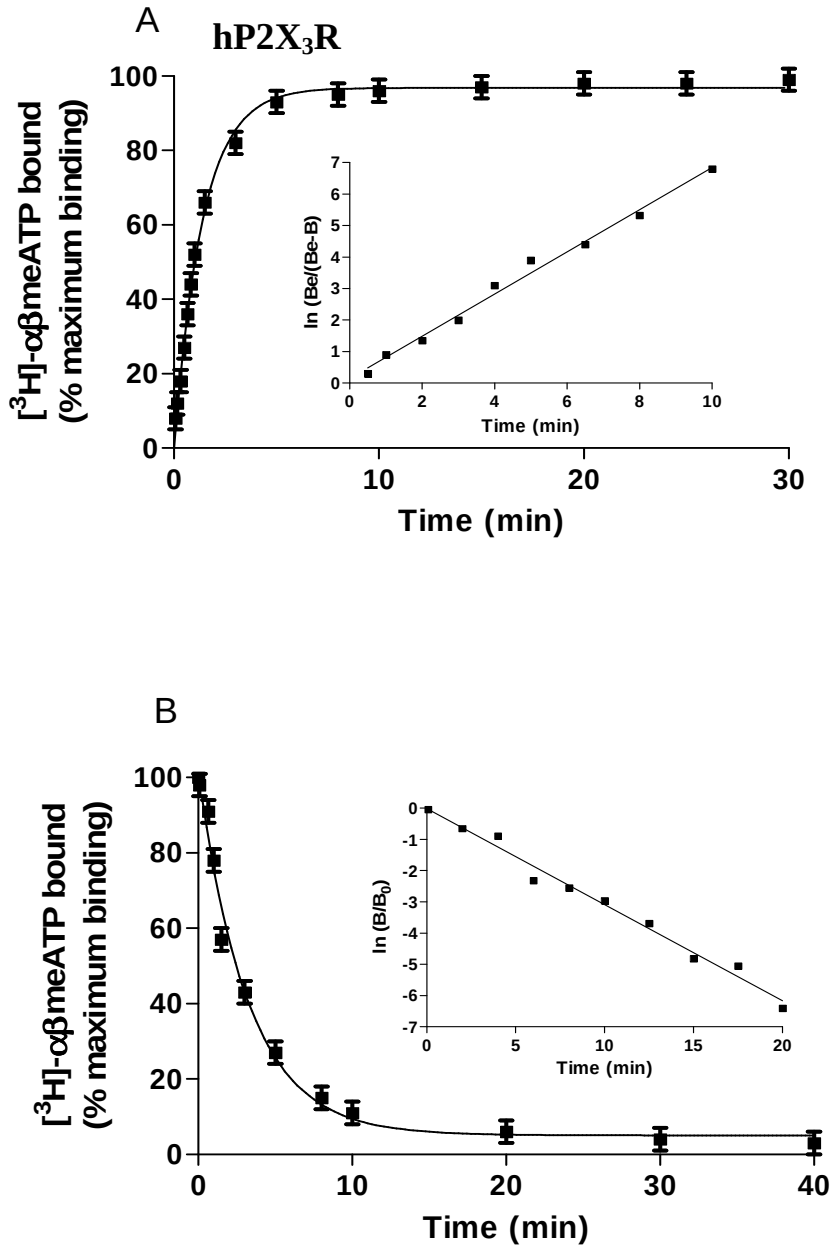


Figure 4.4: Saturation curves of [^{3}H]αβmeATP to HEK-293-hP2X$_1$ membranes at 5 and 25 °C (A) and relative Scatchard plot (B).

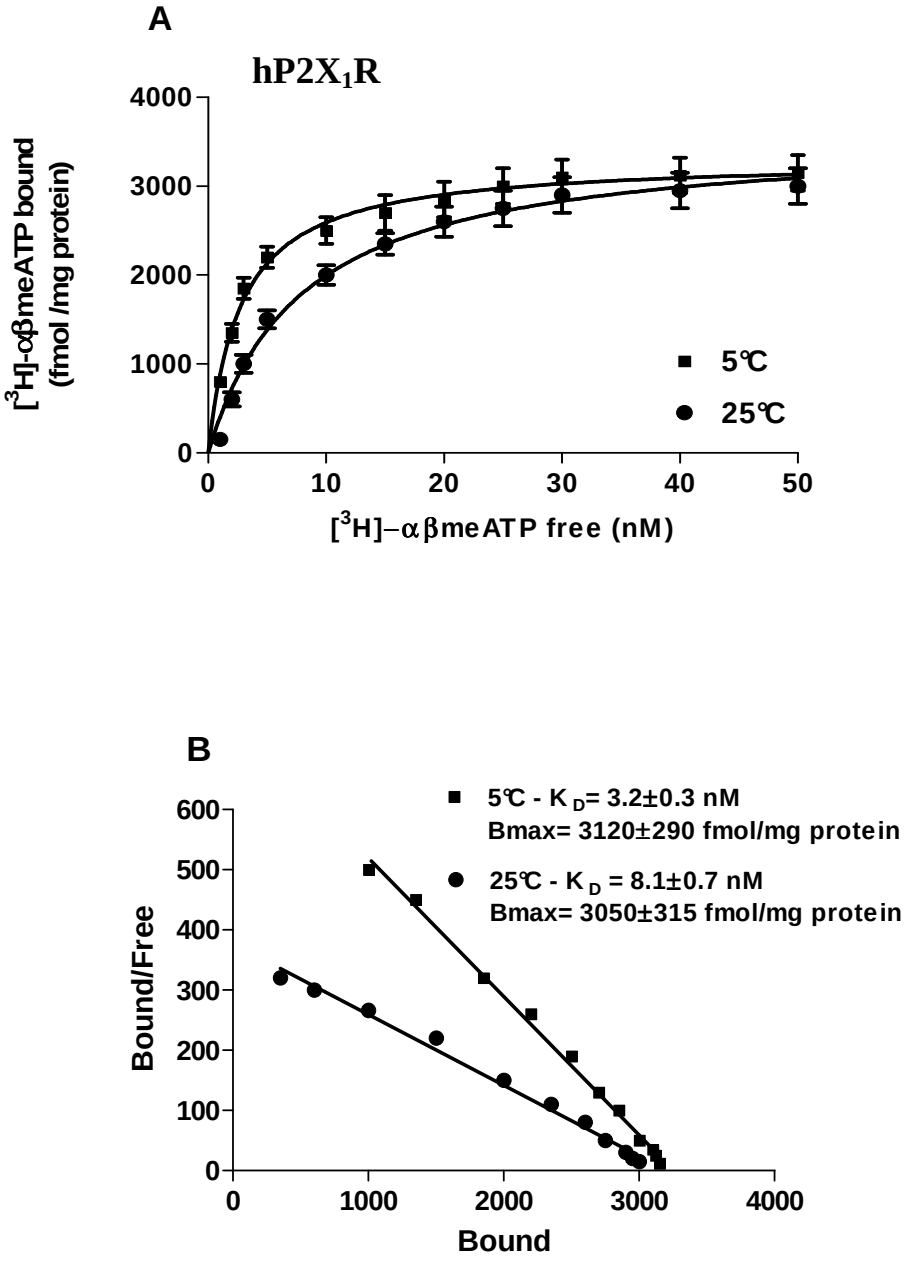


Figure 4.5: Saturation curves of [^{3}H]αβmeATP to HEK-293-hP2X$_3$ membranes at 5 and 25 °C (A) and relative Scatchard plot (B).

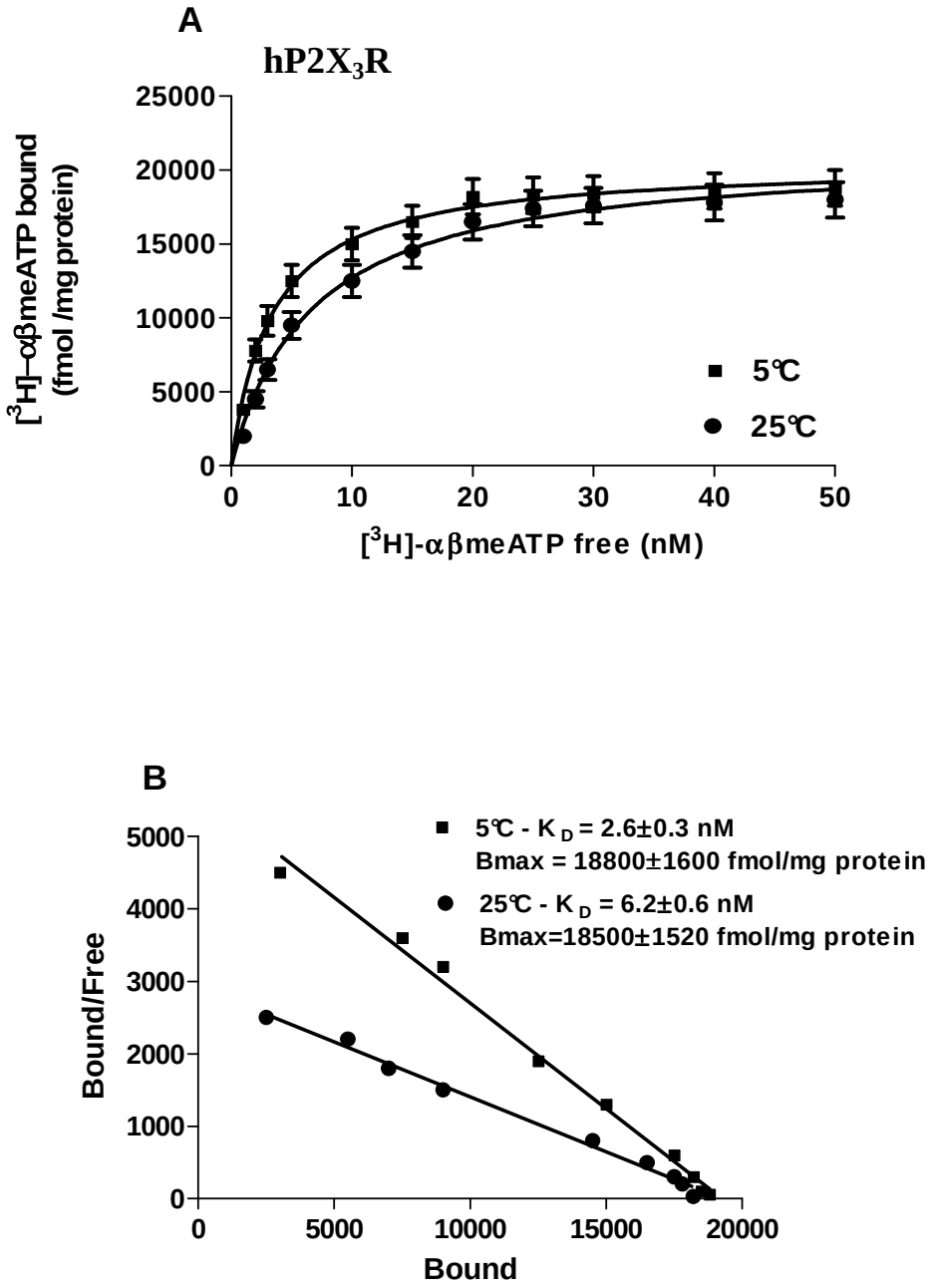


Figure 4.6: Competition curves of specific [^{3}H]αβmeATP binding to HEK-293-hP2X$_1$ by purinergic agonists (A) and antagonists (B).

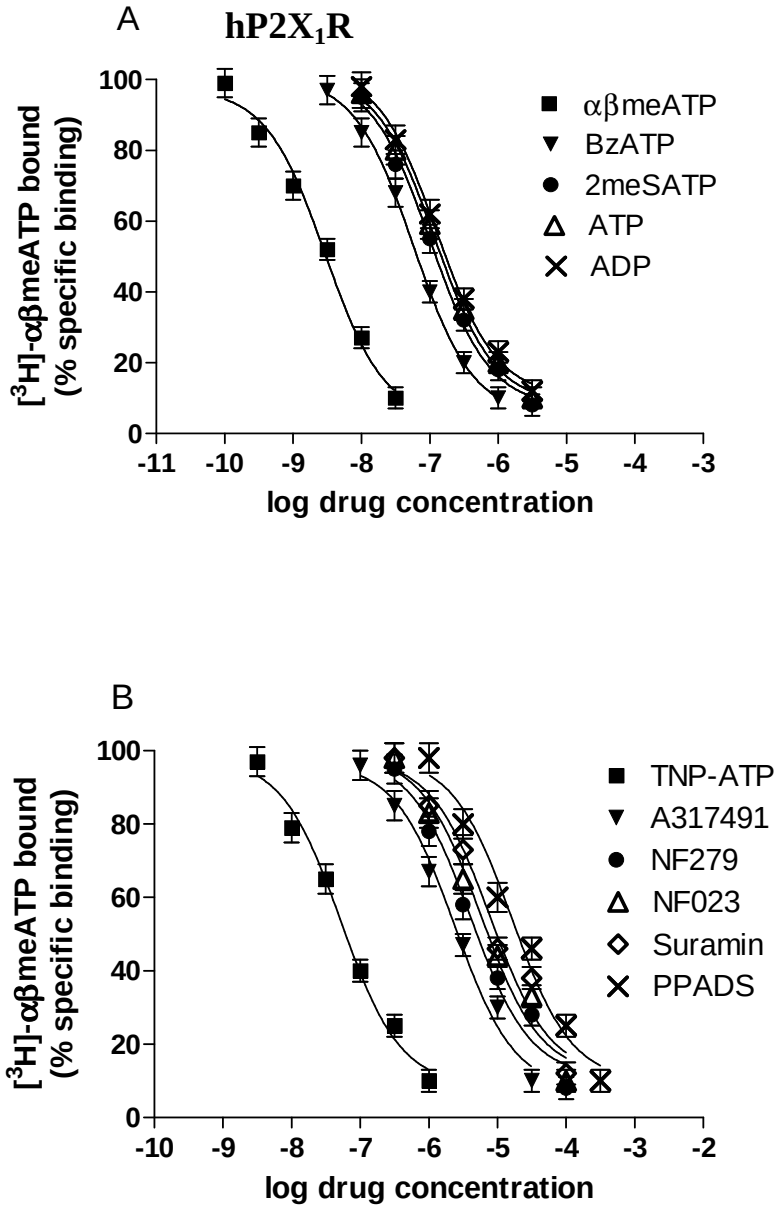


Figure 4.7: Competition curves of specific [^{3}H]αβmeATP binding to HEK-293-hP2X$_3$ by purinergic agonists (A) and antagonists (B).

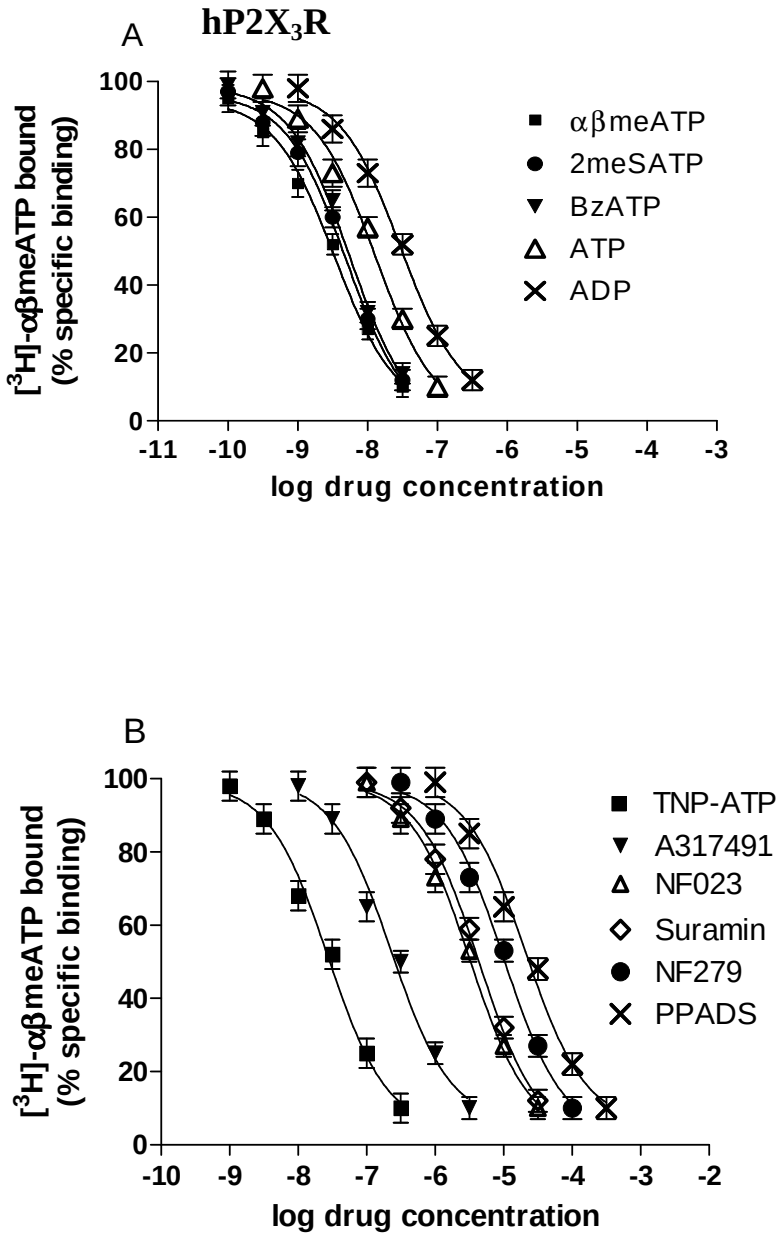


Figure 4.8: van't Hoff plots showing the effect of temperature on the equilibrium binding association constant, K_A, for $P2X_1$ purinergic agonists (A) and antagonists (B).

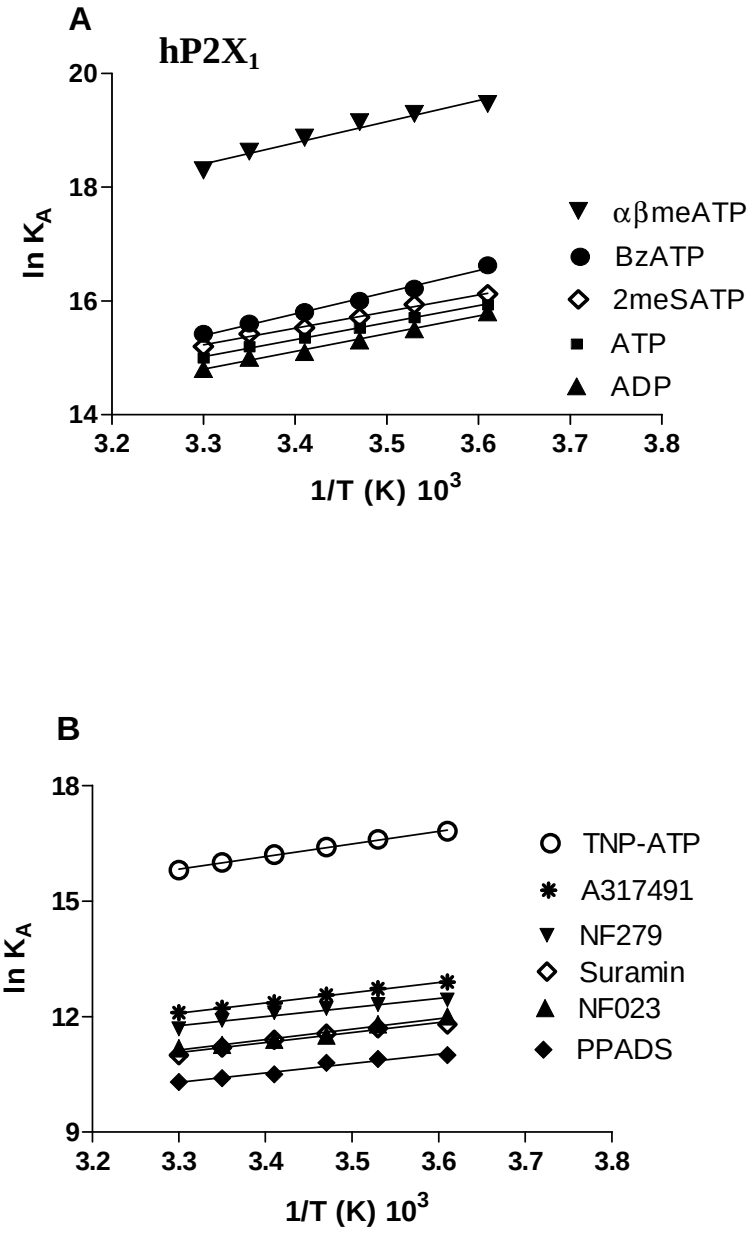


Figure 4.9: van't Hoff plots showing the effect of temperature on the equilibrium binding association constant, K_A, for $P2X_3$ purinergic agonists (A) and antagonists (B).

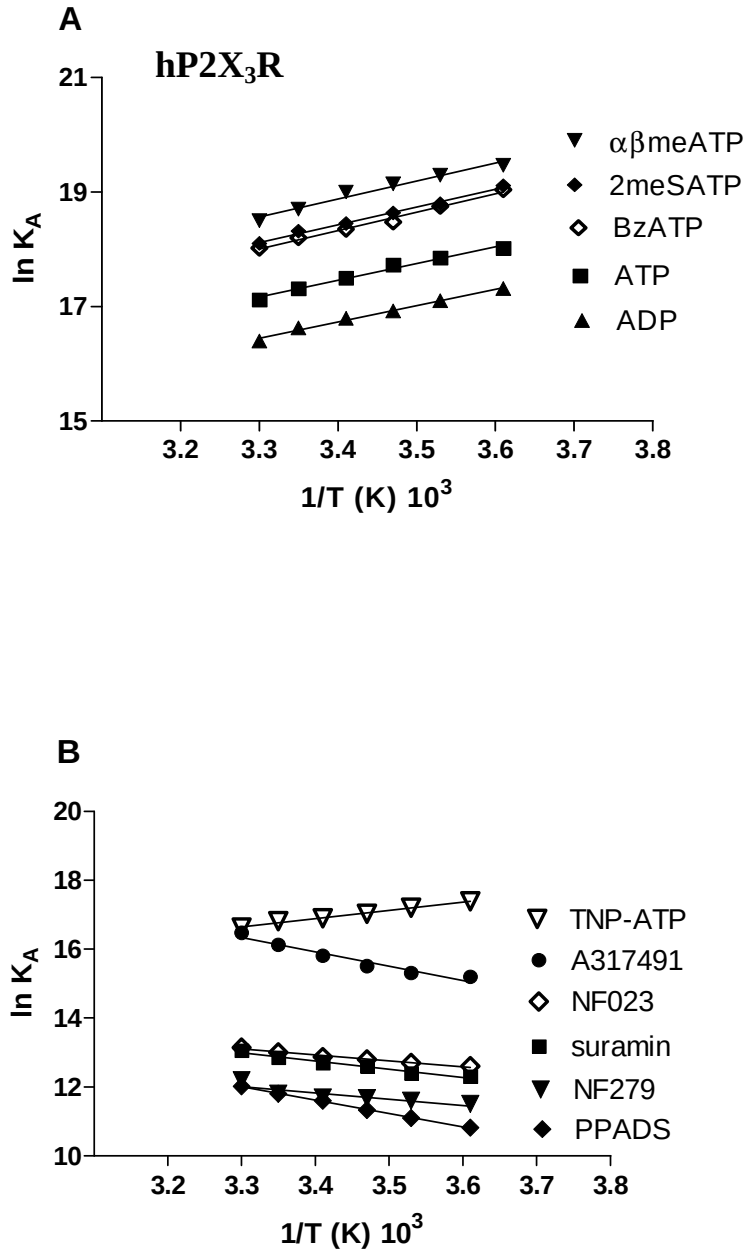
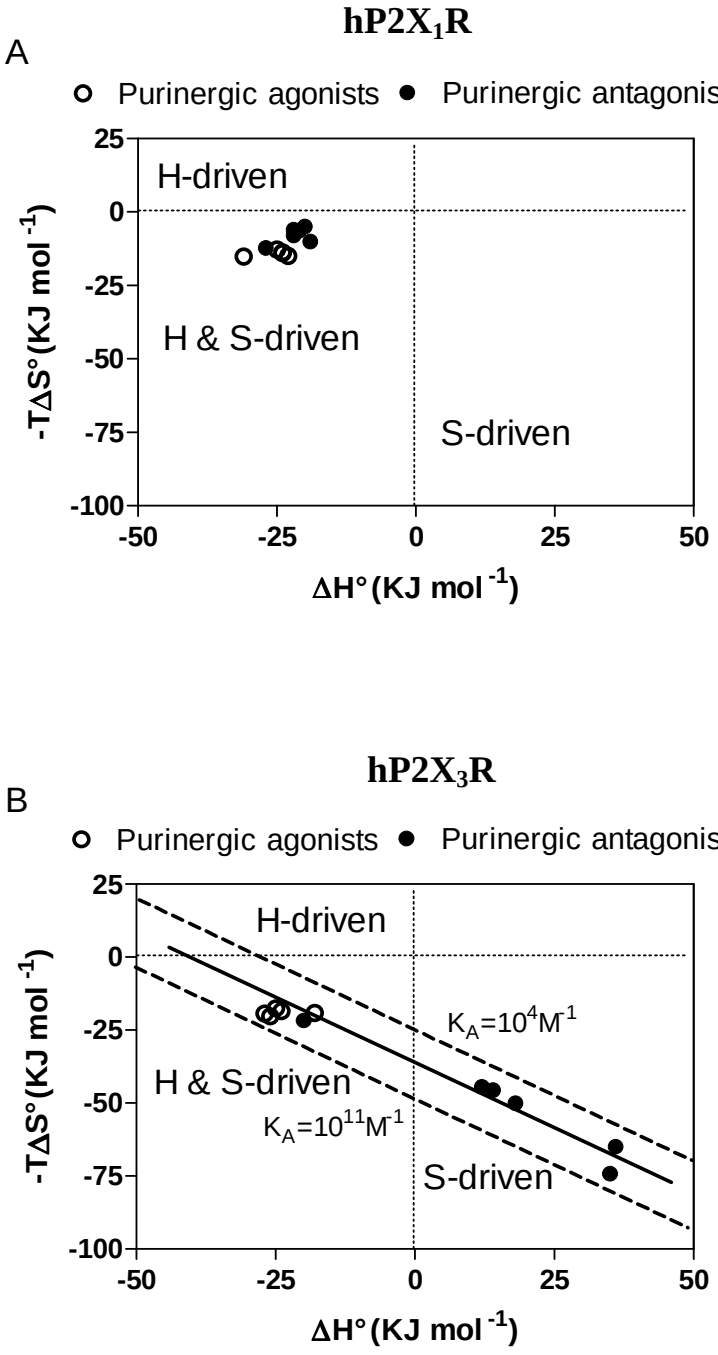


Figure 4.10: Scatter plot showing –TΔS° versus ΔH° values for purinergic ligands studied in HEK-293-P2X$_1$ (A) and HEK-293-P2X$_3$ (B)



DISCUSSION

Thermodynamic parameters have been collected for a remarkable number of ligands at $P2X_1$ and $P2X_3$ receptors, including full, partial and inverse agonists or antagonists (Borea et al., 2000). The information provided by these data could be useful from a pharmacological point of view to discover new thermodynamic relationships related to drug-receptor interactions and their molecular mechanisms (Gilli et al., 1994; Grunwald and Steel, 1995; Raffa, 2001). In the last few years, it has been reported that equilibrium standard enthalpy ($\Delta H°$) and entropy ($\Delta S°$) values of drug interaction with a defined receptor can often give a simple "in vitro" way to discriminate the capability of the drug to interfere with the signal transduction pathways (Borea et al., 1996, 1998, 2000; Merighi et al., 2002; Gilli et al., 2005; Lorenzen et al., 2000). This phenomenon, called "thermodynamic discrimination" has been evaluated for various membrane and cytoplasmatic/nuclear receptors. Six G-protein coupled receptors such as β-adrenergic, D_2 dopamine, $5HT_{1A}$ serotonin and A_1, A_{2A} and A_3 ARs subtypes were studied and four out of six of these were thermodynamically discriminated (Borea et al., 1998, 2000; Merighi et al., 2002). In addition four ligand-gated ion channel receptors such as glycine, $GABA_A$, $5HT_3$ serotonin and nicotinic membrane receptors were analyzed and all resulted discriminated from a thermodynamic point of view (Maksai, 2001; Gessi et al., 1999, Borea et al., 2004). Finally, the cytoplasmatic receptor for glucocorticoid hormones and three cytoplasmatic steroid/nuclear estrogen, progesterone and androgen receptors were investigated and three of these were discriminated (Gilli et al., 2005). All these data suggest an intercorrelation between specific binding and the variation of water molecules present to receptor surfaces. In addition, based on the thermodynamic compensation a general model of drug-receptor interaction has been proposed. In this model the solvent molecules do not modify the intrinsic values of the affinity constant (K_A) of the drug-receptor interaction because the standard free energy for solvent reorganization can be near to zero and the values of binding parameters are due to specific features of the ligand and receptor in the binding process and not by the solvent (Grunwald and Steel, 1995). On the other hand, $\Delta H°$ and $-T\Delta S°$ values are related to the rearrangements occurring during the binding, in the solvent-drug and solvent-receptor interfaces (Grunwald and Steel, 1995). It seems

reasonable to assume that solvent effects might be responsible for the in vitro thermodynamic discrimination between agonists and antagonists. From this background, one of the most significant results of this paper is the presence of the linearity of van't Hoff plots for P2X purinergic receptors similarly to what verified for other membrane receptors showing that ΔH° and ΔS° values are independent of temperature and obtained by linear van't Hoff plots. Van't Hoff plots turn out to be linear for all compounds considered implying that the value of ΔH° is not significantly affected by temperature variation in the range investigated. The second result concerns the interdependence of ΔH° and -TΔS° values for the $P2X_3$ purinergic receptors where all the experimental points appear to be arranged along a same diagonal line according to the equation: -TΔS° (kJ/mol at 278 K) = - 37.8 (± 2) –0.80 (± 0.05) ΔH° (kJ/mol) (n=11, r= 0.985, $p<0.0001$). This equation is of the form $\Delta H° = \beta \Delta S°$ which is expected for a case of enthalpy-entropy compensation with a compensation temperature of 278 K (Borea et al., 1996, 1998, 2000; Merighi et al., 2002; Gilli et al., 2005). The enthalpy-entropy compensation phenomenon has been attributed for drug-receptor interactions to the solvent reorganization (Grunwald and Steel, 1995). While ΔG° values are most probably determined by the features of the ligand-receptor binding process, ΔH° and -TΔS° values appear strongly affected by the rearrangements occurring in the solvent (Tomlinson and Steel, 1983). As it can be seen from the data plotted in Figure 4.10, all experimental points are arranged on the same diagonal band encompassed between the two dashed lines which represent the *loci* of points defined by the limiting K_D values of 100 μM and 10 pM. This band is the expression of the enthalpy-entropy compensation phenomenon probably due to drug-receptor interactions, to the solvent reorganization that accompanies the receptor binding process (Borea et al., 1996, 1998, 2000; Merighi et al., 2002; Gilli et al., 2005). The most remarkable differences between $P2X_1$ and $P2X_3$ receptors are elucidated by the comparison of thermodynamic data. Interestingly, $P2X_1$ and $P2X_3$ purinergic receptors have a different thermodynamic behaviour as demonstrated by the fact that agonists and antagonists for $P2X_1$ receptors show similar enthalpy and entropy values. On the contrary, $P2X_3$ receptors can be considered thermodynamically discriminated because agonist binding is enthalpy and entropy-driven and antagonist binding is totally entropy-driven. Another observation on purinergic ligands studied is

represented by a higher affinity *versus* $P2X_3$ than *versus* $P2X_1$ receptors. High affinity values, in the nanomolar range, were found for αβmeATP in both purinergic receptors studied. The order of potency for $P2X_3$ receptors of the agonists was: αβmeATP > 2meSATP >BzATP > ATP > ADP. Similar potencies were also observed to $P2X_1$ receptors even if all ligands, with the exception of αβmeATP, showed a lower affinity in comparison with that obtained to $P2X_3$ receptors. It should be noted that other examples of binding experiments have been reported by using different agonist or antagonist radioligands (Jarvis et al., 2004; Michel et al., 1995, 1996; Schafer and Reiser, 1997). It was found that in synaptosomal membranes from rat brain cortex, ATP, 2MeSATP and suramin revealed lower Ki values by using [^{35}S]-ATPαS as a radioligand to label P2X receptors, than those found in our conditions (Schafer and Reiser, 1997). The same authors reported a clear difference between the binding characteristics of different agonists and antagonists using [^{35}S]-ATPαS or [^{3}H]αβmeATP (Schafer and Reiser, 1997). Furthermore, different affinity values were found using [^{3}H]-A317491 to label $P2X_3$ receptors expressed in 132N1 human astrocytoma cells (Jarvis et al., 2004). In particular, the main difference in affinity values compared to our data concern the Ki values of the antagonists. In fact, A317491, TNP-ATP and PPADS revealed a higher affinity using [^{3}H]-A317491 instead of [^{3}H]αβmeATP. On the contrary, the affinity values of the agonists were strictly similar using [^{3}H]-A317491 or [^{3}H]αβmeATP as radioligands. Some reports revealed weak correlation between radioligand binding profiles and the functional activity of various P2 ligands (Yu et al., 1999). Binding and functional studies reported that P2X receptor agonists with high affinity values in the nanomolar range also showed a very low potency (Lambrecht, 2000). In addition, differences in P2X binding parameters could be attributed to various additional factors. In particular it was found that several divalent and trivalent cation salts markedly increase binding of αβmeATP (Michel and Humphrey, 1994). Some discrepancies between our and previous data might be related either to a different radioligand used to reveal P2X purinergic receptors or to a various cells or tissues expressing purinergic receptors. Some differences are also present by using human or rat purinergic tissues suggesting a species diversity despite their high homology (Michel et al., 1996). In our experimental conditions suramin analogs such as NF 023 and NF 279 revealed affinity values in the micromolar range for

both purinergic subtypes even if they have been reported be selective for $P2X_1$ receptors (Burnstock, 2007). This is probably due to the various experimental conditions used and may also be complicated by the fact that functional P2X ligand-gated ion channels exist as oligomeric combinations with specific subunit arrangements (North, 2002; North and Surprenant, 2000; Jacobson et al., 2002; Burnstock, 2007). Interestingly, TNP-ATP a typical nucleotide with a ribose-substituted trinitrophenyl group, defined to be an antagonist in functional assays, behaves in our experimental thermodynamic conditions as an agonist. It is possible to hypothesize that TNP-ATP could interact with the site occupied by the agonists. Another possible explanation could be that the binding of TNP-ATP is present to an allosteric site on a large extracellular region of the receptor representing a common domain that interacts with the strongly electronegative trinitrophenyl mojety (Burgard et al., 2000; Virginio et al., 1998). More generally, the present study demonstrates that thermodynamic parameters reflect a common mechanism of ligand-receptor interaction and emphasizes the possibility to obtain information about the agonist-antagonist discrimination by simple *in vitro* binding experiments. Thus, analysis of thermodynamic data of drug-receptor interactions appears to be an effective tool for investigating, at a molecular level, the role played during the binding of the ligands. These novel research could be of interest in the identification of novel and potent $P2X_1$ and $P2X_3$ purinergic ligands.

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CHAPTER 5:

Pharmacological characterization of $P2X_1$ and $P2X_3$ purinergic receptors in bovine chondrocytes.

INTRODUCTION

Chondrocytes within articular cartilage are responsible for the synthesis and degradation of the extracellular matrix, which in healthy tissue provide mechanical functionality. In degenerative diseases such as OA and RA there is a net loss of cartilage because the rate of catabolism exceeds the rate of synthesis and deposition of the macromolecular components of the extracellular matrix (Leong et al., 1994). Several chondrocyte activities are associated to cartilage damage, and include the up-regulation of nitric oxide (NO) and lipid inflammatory mediators such as prostaglandins (PGE), as well as the increased production of matrix-degrading enzymes (De Mattei et al., 2002). Growing evidence suggests that extracellular nucleotides, might play important roles in the regulation of cartilage metabolism (Hoebertz et al., 2003; Picher et al., 2003). Extracellular nucleotides have been shown to enhance growth factor-induced proliferation of chondrocytes (Kaplan et al., 1996). Further it has been reported that ATP and ADP can stimulate the production of cartilage inflammatory mediators such as PGE by cultured human chondrocytes, which is enhanced by the pro-inflammatory cytokines IL-1β, IL-1α and TNF- α (Caswell et al., 1991, 1992; Koolpe et al., 1999). ATP can promote cartilage resorption through the breakdown of proteoglycans and the release of glycosaminoglycans (Leong et al., 1994; Brown et al., 1997). On the other hand, it has been also reported that the release of ATP and the activation of purinergic pathways induced by physiological mechanical stimulation can be involved in beneficial effects on cartilage, including the up-regulation of proteoglycan synthesis and the downregulation of inflammatory mediators such as NO (Graff et al., 2000; Kono et al., 2006; Millward et al., 2000, 2004; Chowdhury and Knight, 2006). These previous studies indicate the involvement of purinergic pathways in the modulation of cartilage metabolism, however, to date, the pharmacological characterization of purinergic receptors and the effects of nucleotides in chondrocytes have not been investigated in detail. Several pharmacological studies on transduction mechanisms and molecular biology indicate the basis for subdivision of P2 receptors into P2X and P2Y families (North, 2002; Burnstock, 2004). In particular, seven subtypes of P2X receptors and eight subtypes of P2Y receptors are currently recognized (Abbracchio et al., 2006). P2X purinergic

receptors are membrane ligand-gated ion channels that open in response to the binding of extracellular ATP which represents an important local regulatory factor of the inflammatory response. P2X receptors are abundantly distributed and functional responses have been described in neurons, glia, epithelia, endotelia, bone, muscle and hemopoietic tissues (North, 2002). Over the last few years attempts to characterize endogenous and recombinant P2X purinergic receptors using radioligand binding techniques have had limited success, primarily due to a lack of selective radioligands (North, 2002; Jacobson et al., 2002). The availability of [^{3}H]-α,βmeATP as a selective radioligand to study P2X purinoceptors presents a very good tool for determining the distribution of these receptors and their binding parameters (Michel et al., 1995). In addition, to better characterize the ligand-receptor interaction a thermodynamic analysis of human $P2X_1$ and $P2X_3$ purinergic receptors expressed in HEK293 cells has been performed (Varani et al., 2008a). Numerous thermodynamic studies have reported that the discrimination of the agonists and antagonists occur in different classes of membrane or intracellular receptors confirming the important role of the thermodynamic parameters to clarify the mechanisms of liganderecеptor binding (Borea et al., 1996, 1998, 2000, 2004; Merighi et al., 2002a). From the current background, the aim of this study was to investigate the presence of P2X receptors in bovine chondrocyte membranes by using western blotting, immunohistochemistry and saturation binding assays. Competition binding experiments and thermodynamic analysis with typical purinergic ligands were also performed to characterize, from a pharmacological point of view, P2X purinergic receptors expressed in bovine chondrocytes. The capabilities of the same purinergic ligands to modulate chondrocyte catabolic activities were evaluated by analyzing the effects on NO release and PGE_2 production, both in the absence and in the presence of IL-1.

MATERIALS AND METHODS

Human $P2X_1$ and $P2X_3$ purinergic receptors expressed in HEH-293 cell culture and membrane preparation

HEK293-h$P2X_1$ and HEK293-h$P2X_3$ cells were kindly provided by Prof A. Surprenant (Institute of Molecular Physiology, University of Sheffield, Sheffield,

England, UK). In particular, HEK293-hP2X$_1$ and HEK293-hP2X$_3$ cells were generated by transformation of human embryonic kidney cell cultures and stably expressed purinergic receptors (Virginio et al., 1998). These cells were grown adherently and maintained in DMEM/F12, containing 10% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml) and Geneticin (G418, 0.2 mg/ml) at 37°C in 5% CO_2 /95% air. For membrane preparation, the culture medium was removed, the cells were washed with PBS and scraped in ice-cold hypotonic buffer. The cell suspension was homogenized and centrifuged for 30 min at 100,000 g. The membrane pellet was resuspended in Tris HCl 50 mM, $CaCl_2$ 4-mM, pH 7.4 and frozen at -80°C.

Chondrocytes culture and membrane preparation

Bovine articular cartilage derived from the metacarpophalangeal joints of 14-18 month-old animals (Limousin breed). Chondrocytes were isolated from cartilage fragments obtained from the weight-bearing region of the articular surface (Pezzetti et al., 1999; De Mattei et al., 2004). Briefly, the cartilage was dissected out and cut into small pieces that were subjected to a sequential digestion in DMEM/F12 (1:1) medium (Gibco-Invitrogen, Paisley, UK) with pronase from Streptomyces griseus (Calbiochem, Darmstadt, Germany) for 90 min and collagenase P from Clostridium histolyticum (Roche, Indianapolis, USA) for 12 h. The resulting suspension was filtered to remove undigested cartilage and chondrocytes were recovered by centrifugation, counted and plated at high density (150,000/cm^2) in 75-cm^2 flasks and in multiwells (1.6 cm the diameter of each well). Chondrocytes were cultured in DMEM/F12 supplemented with 10% FBS and antibiotics (penicillin 100 U/ml, streptomycin 0.1 mg/ml) (GibcoeInvitrogen, Paisley, UK). Only chondrocytes without subculturing and maintained in culture for 1 week were used in the binding and functional experiments. In chondrocyte cultures the presence of aggrecan and type II collagen and the absence of type I collagen were shown by immunohistochemistry indicating the maintenance of the chondrocyte phenotype (Varani et al., 2008b). For membrane preparation, the culture medium was removed and the cells were washed with PBS and scraped off T75 flasks in ice-cold hypotonic. The cell suspension was homogenized, centrifuged for 30 min at 100,000 g and used in saturation and competition binding experiments.

Western blotting analysis for $P2X_1$ and $P2X_3$ receptors

Bovine chondrocytes in comparison with HEK293-h$P2X_1$ and HEK293-h$P2X_3$ cells were harvested and washed with ice-cold PBS containing sodium orthovanadate 1 mM, 4-(2-aminoethyl)-benzenesulfonyl fluoride 104 mM, mM aprotinin 0.08, leupeptin 2 mM, bestatin 4 mM, pepstatin A 1.5 mM, E-64 1.4 mM. Then cells were lysed in Triton lysis buffer and the protein concentration was determined using bicinchoninic acid (BCA) protein assay kit (Pierce). Aliquots of total protein sample (50 mg) were analyzed using polyclonal antibodies for human $P2X_1$ and $P2X_3$ purinergic receptors (Neuromics Antibodies, 1 mg/ml dilution) and for GAPDH (Novus Biologicals) (Merighi et al., 2002b). Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies (1:2000 dilution). Specific reactions were revealed with Enhanced Chemiluminescence Western blotting detection reagent (Amersham Biosciences).

Immunohistochemistry for $P2X_1$ and $P2X_3$ receptors

The expression of $P2X_1$ and $P2X_3$ receptors was also evaluated by immunohistochemistry in cartilage fragments immediately after dissection from the articular surface (Brady et al., 2004). Briefly, cartilage fragments were fixed in freshly prepared 4% paraformaldehyde (PFA) w/v in PBS, pH 7.2 for 3 h and then transferred to 10% sucrose in PBS and refrigerated overnight. The samples were embedded in optimum cutting temperatures medium and 4-5-mm thickness sections were cut using a cryostat and collected onto clean glass superfrost slides. After washing in PBS, endogenous peroxidase was blocked by incubation with 0.3% w/v hydrogen peroxide in methanol. After a further wash in PBS, the tissue sections were incubated with polyclonal rabbit antibodies to $P2X_1$ and $P2X_3$ receptors (Neuromics Antibodies, 1:500) overnight. Stain was obtained by developing with ultraystain polyvalent horseradish peroxidase (HRP) immunostaining kit (Ylem, Rome, Italy), using the procedure recommended by the manufacturer. Negative controls were prepared without primary antibody and nuclei were counterstained by hematoxylin.

Saturation and competition binding assays

Saturation binding experiments of [^{3}H]-α,β-meATP (from 0.1 to 50 nM) to the membranes previously obtained (100 mg of protein/assay) were performed by incubating from 40 min at 5°C to 90 min at 30°C according to the results of previous time-course experiments. Competition experiments of 3 nM [^{3}H]-α,β-meATP were performed in duplicate in test tubes containing Tris HCl 50 mM, $CaCl_2$ 4 mM buffer, 100 ml of membranes and at least 8-10 different concentrations of $P2X_1$ and $P2X_3$ agonists or antagonists studied. ATP, ADP, α,β-meATP, BzATP, TNP-ATP, suramin, NF023, PPADS, A317491 were obtained from Sigma-Aldrich Advanced Sciences (Milan, Italy). NF279 and 2-meSATP were obtained from Tocris Cookson Ltd (Bristol, UK). Non specific binding was determined in the presence of 10 mM α,β-meATP (Froldi et al., 1997). Bound and free radioactivity were separated by rapid filtration through Whatman GF/B glass-fiber filters using a Brandel instrument. The filter bound radioactivity was counted on a Scintillation Counter Tri Carb Packard 2500 TR (efficiency 57%). The protein concentration was determined according to a Bio-Rad method with bovine albumin as reference standard (Bradford, 1976).

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: $\Delta G° = \Delta H° - T\Delta S°$. The standard free energy was calculated as $\Delta G° = -RT\ln K_A$ at 298.15 K, the standard enthalpy, $\Delta H°$, from the van't Hoff plot $\ln K_A$ versus (1/T) (the slope of which is $-\Delta H°/R$) and the standard entropy as $\Delta S° = (\Delta H° - \Delta G°)/T$ with T = 298.15 K and R = 8.314 J/K/mol (Jacobson et al., 2002; Merighi et al., 2002; De Mattei et al., 2004). K_A values were obtained from [^{3}H]-α,β-methyleneATP saturation experiments in bovine chondrocyte membranes carried out at 0, 10, 15, 20, 25 and 30°C in a thermostatic bath assuring a temperature of ± 0.1°C.

Pharmacological treatments of bovine chondrocytes

In functional assays including the analysis of PGE_2 and NO release, chondrocytes isolated from cartilage, plated at high density (150,000/cm^2) in multiwells and

maintained in culture for 1 week were used. At the beginning of the experiments (time 0), medium was changed in all cultures. To test the effects of purinergic agonists cells were incubated in complete medium containing increasing doses of ATP (10, 100, 500 μM) and α,β-meATP (1, 10, 100 μM). In a second series of experiments ATP (10, 100, 500 μM) and α,β-meATP (1, 10, 100 μM) were added to medium containing 50 ng/ml Recombinant Human IL-1β (Preprotech INC, Rocky Hill, NJ, USA), used to activate inflammatory activities in chondrocytes. To evaluate the capability of A317491 to antagonize the effects of the purinergic agonists, 1 mM of this antagonist was added to the treated cultures. All treatments were made up in triplicate wells for 24 h. At the end of treatment, the culture medium was removed from the wells and stored at -80°C for subsequent determination of PGE_2 and NO release. The monolayers were solubilized in sodium hydroxide and protein concentration was evaluated (Bradford, 1976).

NO assay

The release of nitrite, a stable breakdown product of NO was measured as an indicator of NO synthesis. At the end of the 24 h of treatment period, nitrite concentration was determined in the conditioned media according to Greiss method (Green et al., 1982.) Briefly, culture medium was mixed with an equal volume of the Greiss reagent and absorbance was measured at 550 nm. Nitrite concentration was calculated from a standard curve of sodium nitrite.

PGE_2 assay

The concentration of PGE_2 was measured using a commercially available competitive enzyme immunoassay (PGE_2 ASSAY, R&D Systems, Inc., MN, USA). Briefly, medium was diluted threefold in Calibrator Diluent (RD5-39) included in the kit and 100 μl of diluted sample or standard were added to each well of a microplate coated with goat antimouse antibody. Subsequently, horseradish peroxidase (HRP)-labeled PGE_2 and a mouse monoclonal antibody to PGE_2 were added to each well and the plate was incubated for 2 h and washed. The substrate solution was added to each well and following 30-min incubation a stop solution containing phosphoric acid was added to each well and the absorbance was measured at 450 nm with a reference of 570 nm. All samples and

standards were assayed in duplicate. PGE_2 production was normalized to the total protein content and expressed as picogram (pg) PGE_2/mg protein.

Statistical analysis

A weighted non linear least-squares curve fitting program Ligand (Munson and Rodbard, 1980) was used for computer analysis of saturation and competition binding experiments. Functional experiments were calculated by non linear regression analysis using the equation for a sigmoid concentration response curve (GraphPAD Prism, San Diego, CA, USA). Analysis of data was done with Student's t test (unpaired analysis). Differences were considered significant at a value of $p<0.01$. All data are reported as mean ± SEM of independent experiments ($n = 3$-4).

RESULTS

Western blotting

Figure 5.1 shows the immunoblot signals of $P2X_1$ and $P2X_3$ purinergic receptors in bovine chondrocytes in comparison with HEK293-h$P2X_1$ and HEK293-h$P2X_3$ cells. $P2X_1$ and $P2X_3$ purinergic receptors are present in bovine chondrocytes showing a minor expression than in HEK293-h$P2X_1$ and HEK293-h$P2X_3$ cells. In addition, in bovine chondrocytes $P2X_1$ receptor expression resulted lower than $P2X_3$ receptor. Figure 5.1 also reports the immunoblot signals of GAPDH used as loading control.

Immunohistochemistry

The immunohistochemistry of purinergic receptors in cartilage slices showed that chondrocytes in all zones of the articular cartilage stained positive for $P2X_3$ receptors con-firming the expression of the receptor in intact cartilage (Figure 5.2A). No signal for $P2X_1$ receptors was observed in cartilage slices (Figure 5.2B). Negative controls, obtained without primary antibodies, did not show any staining.

Saturation and competition binding experiments

Saturation binding experiments in bovine chondrocytes were performed to evaluate affinity (K_D) and receptor density (Bmax) values of P2X purinergic

receptors (Table 5.1, figure 5.3). The binding parameters were determined at various temperatures (0, 10, 15, 20, 25 and 30°C) by using [^{3}H]-α,β-meATP as radioligand at different concentrations. These results demonstrate that K_D values change with temperature and Bmax values appear to be largely independent of it. Computer analysis of the data failed to show a significantly better fit to a two site than to a one site binding model, indicating that only one class of high affinity binding site is present under our experimental conditions. Figure 5.4 shows the dose response curves of [^{3}H]-α,β-meATP binding in bovine chondrocyte membranes by using typical agonists and antagonists (Table 5.2). The order of potency in [^{3}H]-α,β-meATP displacement assays for purinergic agonists was as follow: α,β-meATP > BzATP > 2-meSATP > ATP > ADP. Similarly, the order of potency of antagonists in bovine chondrocyte membranes was as follow: TNP-ATP > A317491 > Suramin > NF023 > NF279 > PPADS.

Thermodynamic analysis

The van't Hoff plots show that the effect of temperature on the equilibrium binding association constants, K_A appears to be essentially linear in the range 4-30°C for the purinergic ligands examined (Figure 5.5). Final thermodynamic parameters calculated for the binding equilibria of the different compounds investigated are reported in table 5.3 Thermodynamic parameters of bovine chondrocytes for agonists show an enthalpy- and entropy-driven binding and the antagonist binding was totally entropy-driven. Interestingly, the compound TNP-ATP that functional experiments reported to be a purinergic antagonist (Burgard et al., 2000) shows a typical agonist behavior with an affinity value and thermodynamic parameters strictly similar to those obtained from other purinergic agonists. Figure 5.6A reports the van't Hoff plot and thermodynamic parameters of the [^{3}H]-α,β-meATP radioligand showing that this binding was enthalpy- and entropy-driven. Figure 5.6B summarizes the results obtained in bovine chondrocytes in the form of $-T\Delta S°$ vs $\Delta H°$ scatter plot (T = 298.15 K) and shows that purinergic antagonists are clustered in the endothermic region ($15 \leq \Delta H° \leq 30$ kJ/mol) with large positive entropy values ($-58.11 \leq T\Delta S° \leq -44.40$ k/J/mol) revealing that their binding is totally entropy-driven. Agonistic binding was enthalpy- and entropy-driven ($-26 \leq \Delta H° \leq -24$ kJ/mol and $-20.26 \leq T\Delta S° \leq -$

11.62 k/J/mol) suggesting that agonists and antagonists in bovine chondrocytes are discriminated from a thermodynamic point of view.

Effects of purinergic ligands on NO production in chondrocyte monolayers

Figure 5.7 illustrates the effects of ATP and α,β-meATP on NO production in bovine chondrocytes in basal condition (Figure 5.7A) and after IL-1β treatment (Figure 5.7B). In the absence of IL-1β, treatment of cells with 500 μM ATP and 100 μM α,β-meATP induced a low but significant increase on basal NO production (Figure 5.7A). In the same experimental conditions described above, ATP until 100 μM concentration and α,β-meATP until 10 μM concentration were not able to modulate NO release. In the presence of IL-1β (Figure 5.7B), both ATP and α,β-meATP induced a dose-dependent increase on NO production, ranging from 11 to 84% with respect to IL-1β -treated cells. The purinergic antagonist A317491 efficiently counteracted the activity of both ATP and α,β-meATP inhibiting NO production to the levels of cells treated with IL-1β alone.

Effects of purinergic ligands on PGE_2 production in chondrocyte monolayers

Figure 5.8 shows the effects of ATP and α,β-meATP on PGE_2 production in bovine chondrocytes in basal condition (Figure 5.8A) and after IL-1β treatment (Figure 5.8B). In the absence of IL-1β, treatment of cells with ATP in the range of concentrations from 10 to 500 μM and α,β-meATP (10 and 100 μM) induced a significant increase on basal PGE_2 production (Figure 5.8A). In the same experimental conditions described above, 1 μM of α,β-meATP was not able to modulate PGE_2 release. In the presence of IL-1β, both ATP (500 μM) and α,β-meATP (100 μM) induced a maximum increase on PGE_2 production, of 137-79%, respectively. ATP at the 100 μM concentration was able to induce slightly but significant PGE_2 release ($p<0.05$; figure 5.8B). The purinergic antagonist A317491 counteracted in a different way the activity of both ATP and α,β-meATP (Figure 5.8B). In particular, A317491 partially reduced ATP-mediated PGE_2 release whilst it completely inhibited α,β-meATP-mediated PGE_2 release.

Table 5.1:Binding parameters, expressed as K_D and Bmax, of [^{3}H]-α,β-meATP in bovine chondrocyte membranes.

[^{3}H]αβmeATP binding	5°C (278 K)	10°C (283 K)	15°C (288 K)	20°C (293 K)	25°C (298 K)	30°C (303 K)
K_D (nM)	3.3±0.3	4.3±0.4	5.5±0.5	6.6±0.6	7.5±0.7	8.4±0.7
Bmax (fmol/mg protein)	4900±420	4950±440	5050±480	5000±470	4800±450	4750±490

Table 5.2: Affinity, expressed as Ki values, of selected purinergic agonists and antagonists in bovine chondrocyte membranes at different temperatures.

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)
Purinergic Agonists	Ki (nM)	Ki (nM)	Ki (nM)	Ki (nM)	Ki (nM)	Ki (nM)
ATP	90±8	120±11	137±15	168±17	200±19	250±22
ADP	120±11	150±12	186±17	205±18	250±20	306±28
αβmeATP	3.0±0.3	4.1±0.3	5.0±0.4	6.1±0.5	6.9±0.6	8.1±0.7
2meSATP	85±8	110±10	130±12	150±15	185±20	220±22
BzATP	80±7	100±9	124±13	145±14	170±16	205±21
Purinergic Antagonists						
Suramin	9000±850	8000±750	7000±650	6500±550	5500±450	5000±400
NF023	11000±850	10000±850	9000±850	8500±850	7500±850	6000±850
TNP-ATP	100±10	135±15	150±16	180±18	230±25	280±20
PPADS	25000±2200	21000±2000	18000±1700	12000±1000	10000±900	9000±800
A317491	950±85	800±70	750±60	600±60	550±50	450±40
NF279	15000±1400	13000±1300	12500±1200	11000±1100	9500±900	8000±700

Table 5.3: Thermodynamic parameters for the binding equilibrium of [^{3}H]-α,β-meATP to bovine chondrocyte membranes of selected purinergic agonists and antagonists.

Ligand	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol/K)
Purinergic Agonists			
ATP	-38.0±3.8	-26±2	39±2
ADP	-37.5±3.7	-24±1	44±3
αβmeATP	-46.4±4.1	-26±2	68±3
2meSATP	-38.2±3.4	-25±1	45±4
BzATP	-38.4±3.6	-25±2	46±2
Purinergic Antagonists			
Suramin	-30.0±3.0	16±1	155±5
NF023	-29.2±2.9	15±1	149±5
TNP-ATP	-37.7±3.5	-27±2	38±3
PPADS	-21±2.0	30±2	195±8
A317491	-35.6±3.4	19±1	184±7
NF279	-28.6±2.8	16±0	150±5

Figure 5.1: Western blotting analysis of bovine chondrocytes for purinergic receptors in comparision with HEK293-hP2X$_1$ and HEK293-hP2X$_3$ cells.

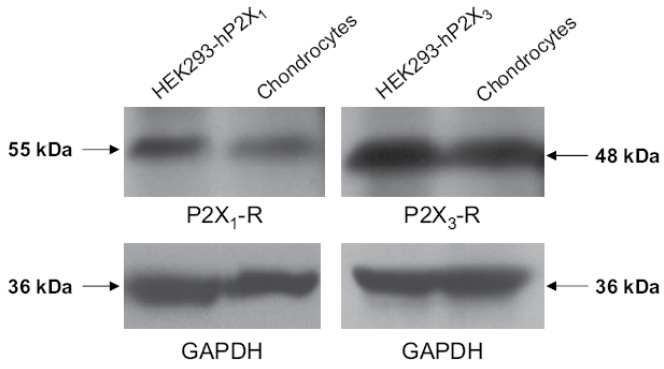


Figure 5.2: Immunohistochemistry of P2X$_3$ receptors (A) and P2X$_1$ receptors (B) in cartilage section. In (B) nuclei are counterstained by hematoxylin.

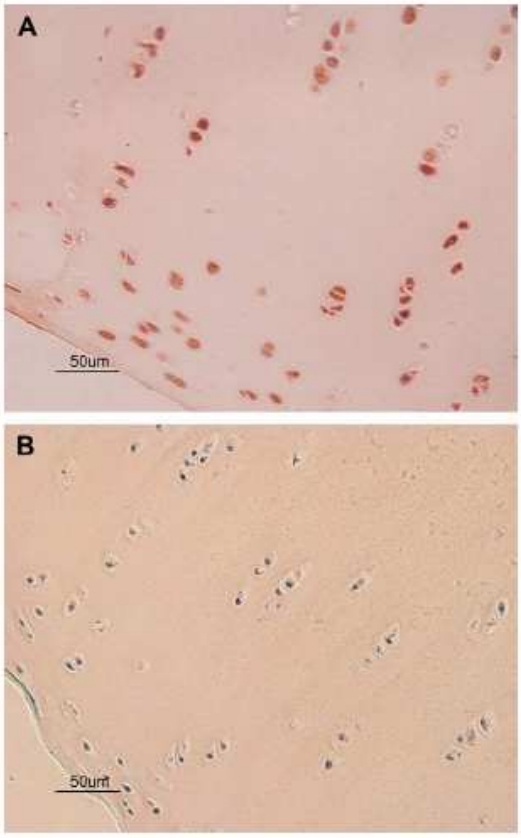
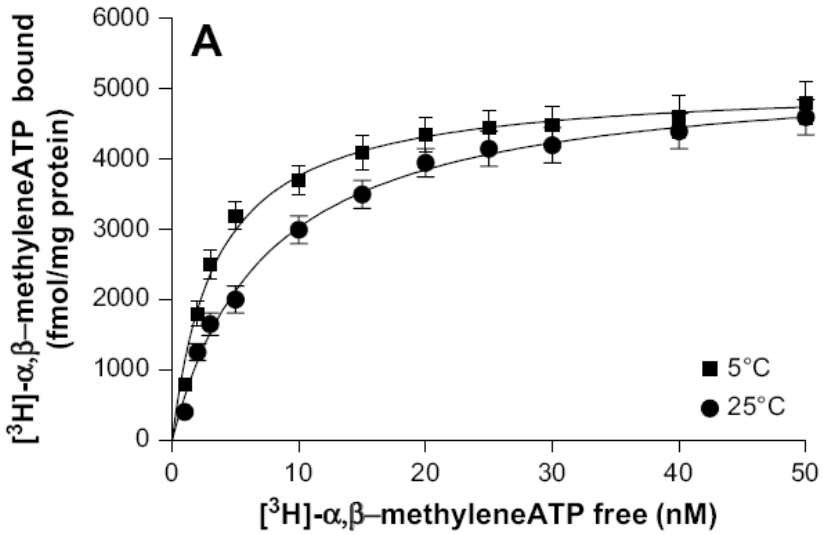


Figure 5.3: Saturation curves of [^{3}H]-α,β-meATP binding to P2X purinergic receptors in bovine chondrocytes at 5°C and 25°C (A) and relative Scatchard plot (B).



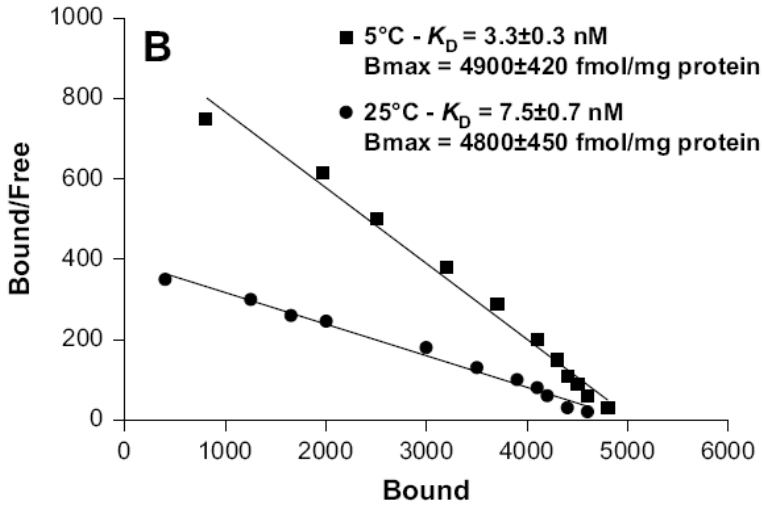


Figure 5.4: Competition curves of typical purinergic agonists (A) and antagonists (B) in bovine chondrocytes.

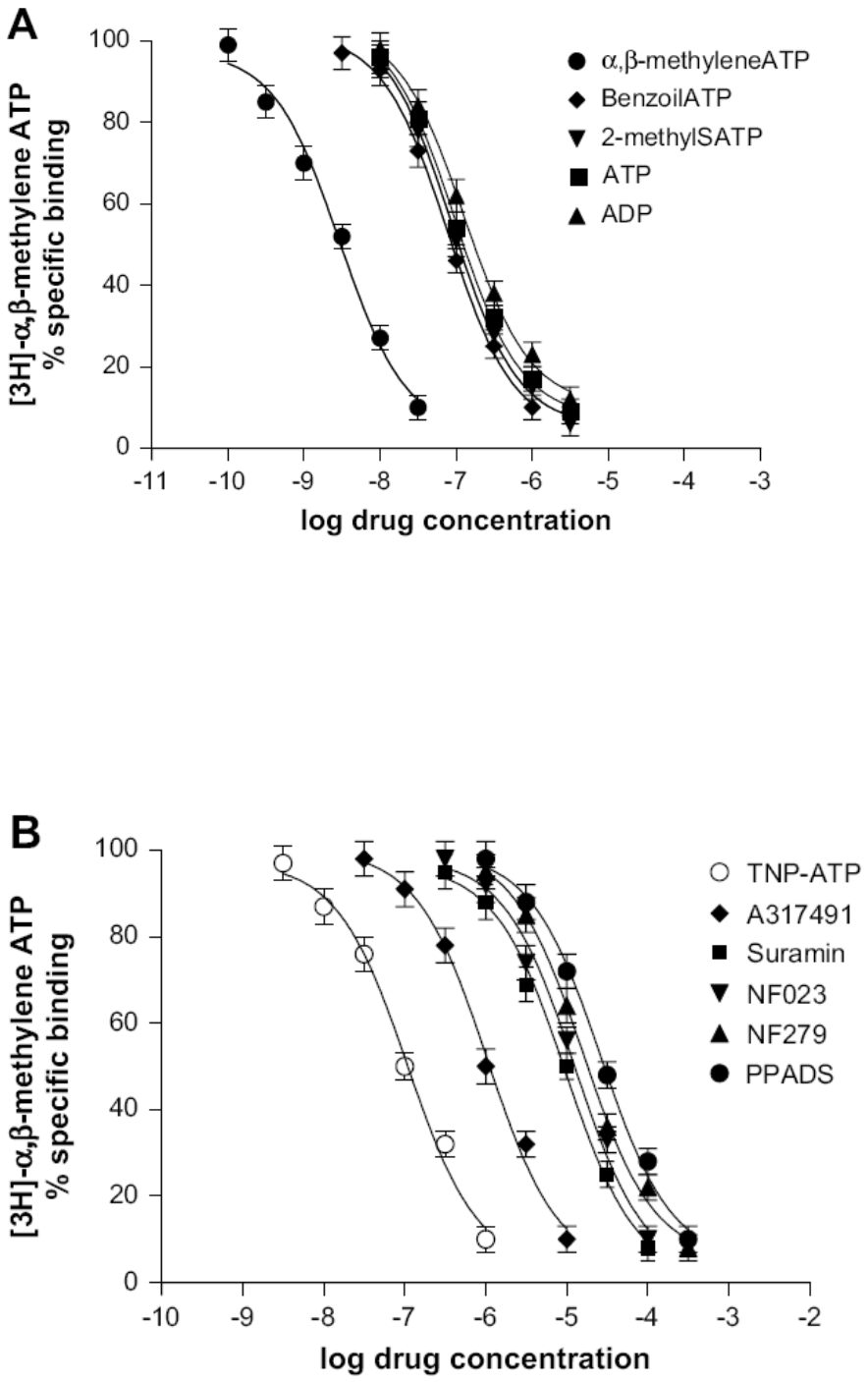
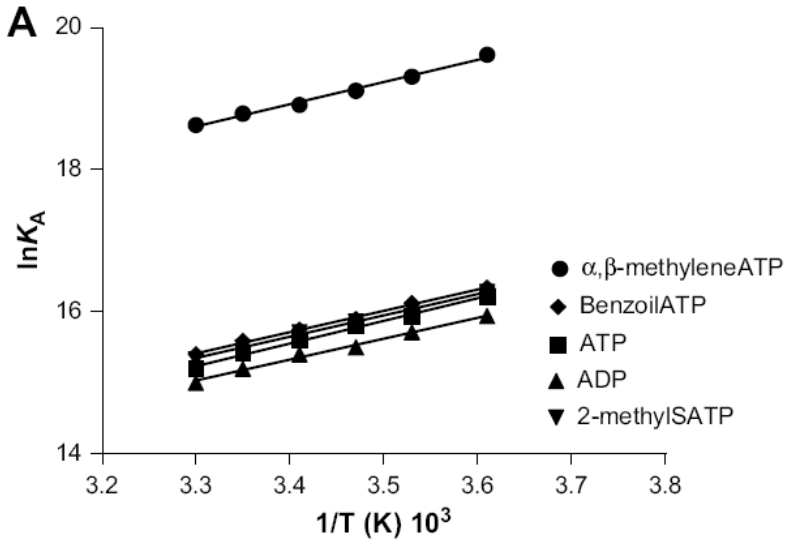


Figure 5.5: Van't Hoff plot in bovine chondrocytes showing the effect of temperature on the equilibrium binding association constant, K_A, for P2X purinergic agonists (A) and antagonists (B).



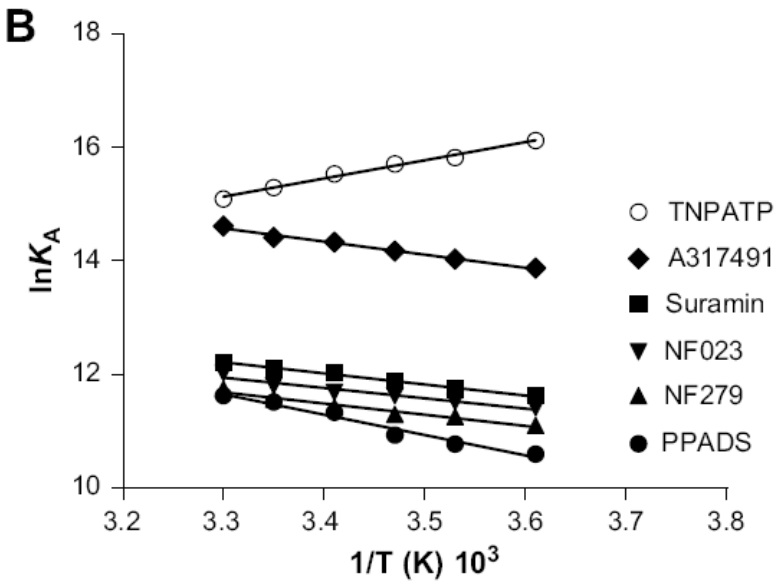


Figure 5.6: Van't Hoff plot in bovine chondrocytes showing the effect of temperature on the equilibrium binding association constant, K_A, of [^{3}H]-α,β-meATP to P2X purinergic receptors (A). Scatter plot showing –TΔS° vs ΔH° values for the purinergic ligands studied (B).

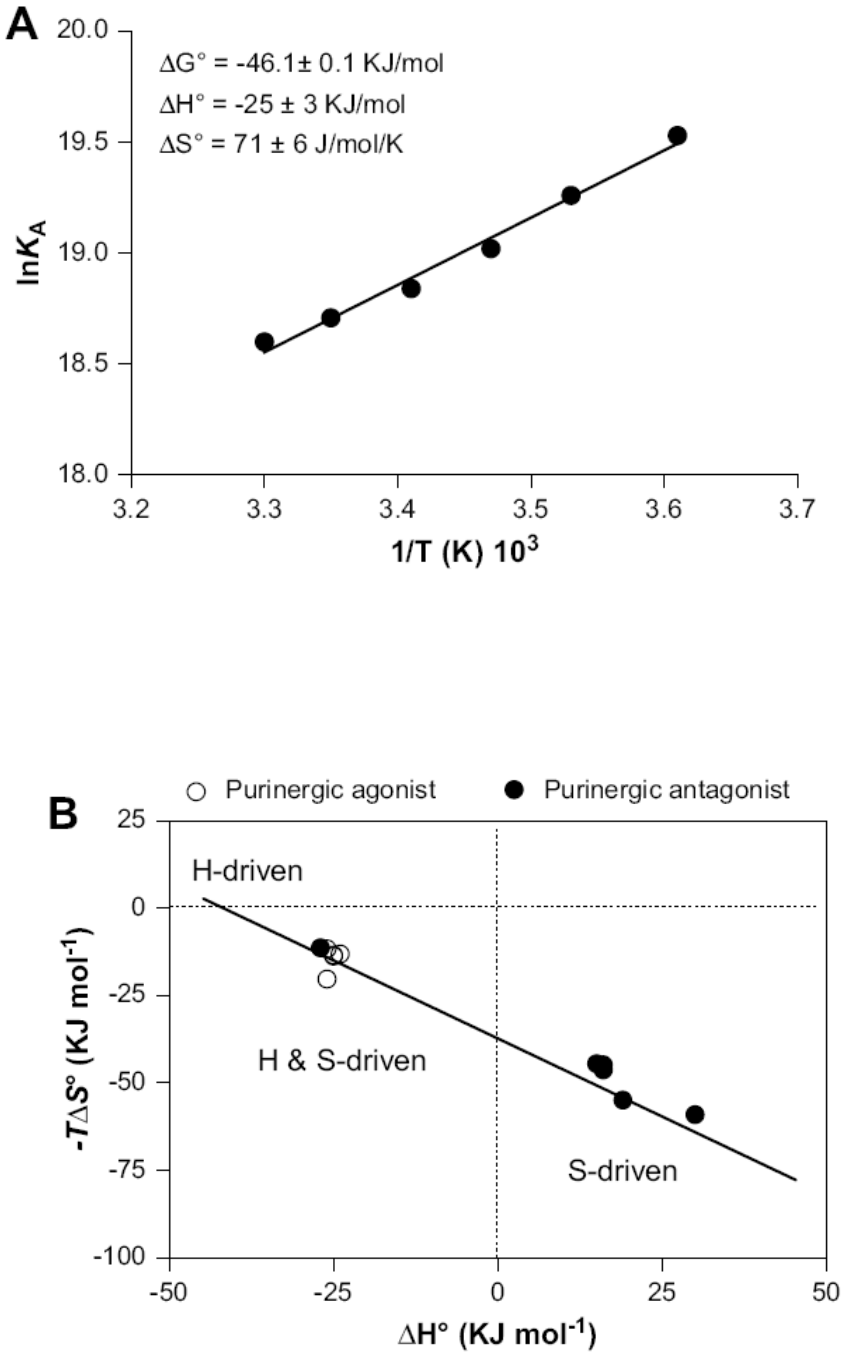
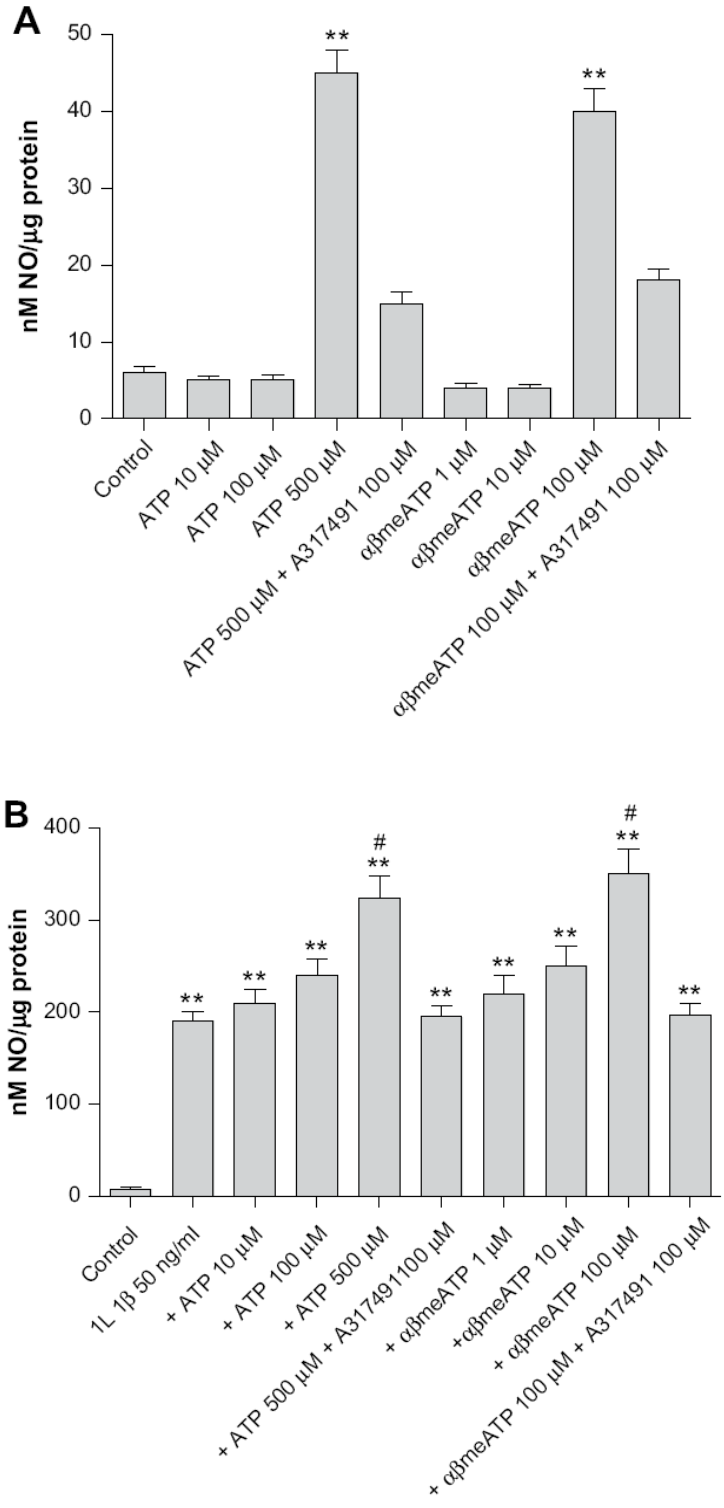
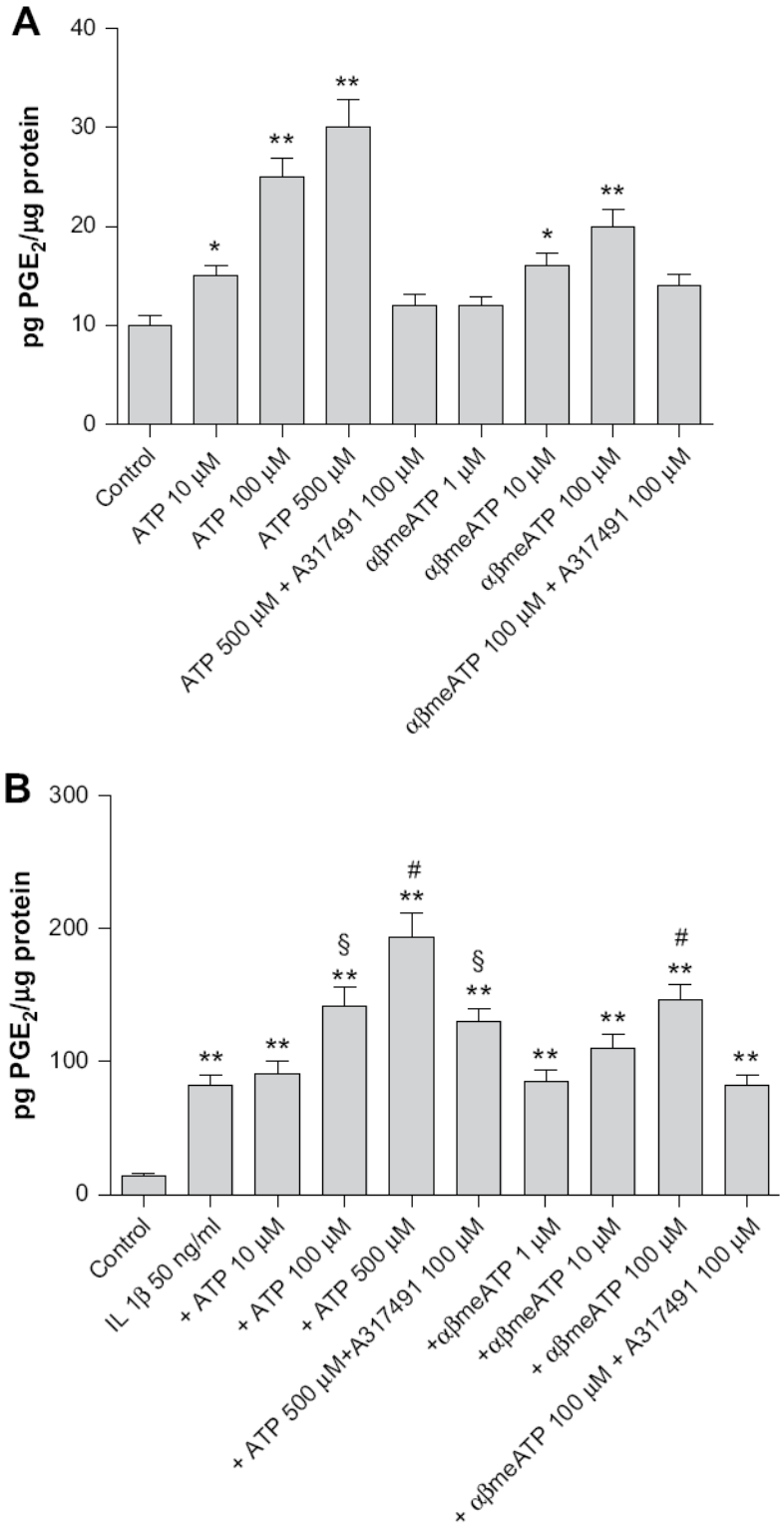


Figure 5.7: Effect of purinergic ligands in the absence (A) and in the presence (B) of IL-1β on NO release in bovine chondrocytes.



** p<0.01 vs Control; # p< 0.01 vs IL-1β 50 ng/ml

Figure 5.8: Effect of purinergic ligands in the absence (A) and in the presence (B) of IL-1β on PGE_2 release in bovine chondrocytes.



** p<0.01 vs Control; * p< 0.05 vs Control; # p< 0.01 vs IL-1β 50 ng/ml; § p< 0.05 vs IL-1β 50 ng/ml

DISCUSSION

The purpose of the current investigation was to document the expression, the binding parameters and the functionality of P2X purinergic receptor subtypes in bovine chondrocytes. The presence of purinergic receptors was investigated through western blotting analysis, immunohistochemistry and saturation binding experiments. Western blotting assays reveal the presence of $P2X_1$ and $P2X_3$ purinergic receptors as reported in figure 5.1 where bovine chondrocytes were analyzed in comparison with HEK293-h$P2X_1$ and HEK293-h$P2X_3$ cells. In both cell types, $P2X_3$ purinergic receptors were present at higher level than $P2X_1$ purinergic receptors. Immunohistochemistry in cartilage slices confirmed the presence of $P2X_3$ purinergic receptors while $P2X_1$ purinergic receptors were not detectable probably due to the low presence of these receptors in this substrate. Another possible hypothesis could be that $P2X_1$ purinergic expression was induced by the in vitro culture conditions. Purinergic receptors were also characterized in saturation binding experiments showing the presence of an high affinity binding site with a K_D of 3.3 ± 0.3 nM and a receptor density (Bmax) of 4900 ± 420 fmol/mg protein. In addition, thermodynamic parameters obtained from the van't Hoff plot indicate that [^{3}H]-α,β-meATP binding to purinergic receptors is enthalpy- and entropy-driven, with a major contribution of the enthalpic component. The information provided by these data could be useful from a pharmacological point of view to discover new thermodynamic relationships linked to ligand-receptor interactions useful to the development of new potential classes of drugs (Gilli et al., 1994, 2005; Raffa, 2001; Lorenzen et al., 2000; Gessi et al., 1999; Maksai, 2001). From the competition binding experiments, performed with the aim to calculate the affinity of the agonists for purinergic receptors, the order of potency was α,β-meATP > BzATP > 2-meSATP > ATP > ADP. The same order of potency was reported at all the temperatures investigated even if the affinity values of the purinergic compounds decreased with the increase of the temperature. The order of potency of the studied purinergic antagonists was TNP-ATP > A31749 > Suramin > NF023 > NF279 > PPADS. Similarly, the order of potency was the same from 5°C to 30°C even if the affinities of the antagonists increased (Ki decreased) with the increase of the temperature. Moreover, in our experimental conditions, purinergic agonists

revealed a higher affinity, in the nanomolar range, when compared to purinergic antagonists that showed an affinity in the low nanomolar-micromolar range. In addition α,β-meATP was the most affine and potent agonist showing an high affinity in the nanomolar range. On the contrary ATP, a typical purinergic endogenous ligand, revealed a lower affinity than α,β-meATP vs P2X purinergic receptors. Collectively, these binding data of affinity are important to identify the specific concentrations of agonists and antagonists to use in functional and molecular in vitro and in vivo experiments. Another purpose of the present study was to investigate from a functional point of view the purinergic P2X receptors in bovine chondrocytes. To this aim, the effects of ATP and α,β-metATP on NO production and PGE_2 release were analyzed. Both NO and PGE_2 are synthesized by chondrocytes (Stadler et al., 1991), and have been implicated as critical inflammatory mediators of cartilage matrix degradation (Stefanovic-Racic et al., 1997; Bankers-Fulbright et al., 1996; Stabellini et al., 2003; Knott et al., 1994). ATP and α,β-meATP were able to increase NO production at micromolar concentration similarly to that reported in literature (Leong et al., 1994; Caswell et al., 1991) and the P2X antagonist A317491 was able to decrease the stimulatory effect mediated by the purinergic agonists investigated. It is well known that IL-1β, a proinflammatory agent which is present in elevated amount in osteoarthritic cartilage and plays a decisive role in osteoarthritis, also increases the production of NO (Stefanovic-Racic et al., 1997). In agreement the treatment of bovine chondrocytes with 50 ng/ml of IL-1β significantly increased NO synthesis. Interestingly, ATP and α,β-meATP were able to further stimulate the production of NO in a dose-dependent manner and A317491 completely inhibited the stimulatory effect mediated by the purinergic agonists. The similar behavior of ATP and α,β-meATP and the complete inhibition of their effects by A317491 indicate the involvement of P2X receptors in the stimulation of NO release. In addition, ATP and α,β-meATP stimulated the production of PGE_2 in a dose-dependent manner, both in the absence and in the presence of IL-1β, indicating a role for P2X in PGE_2 release. Interestingly, ATP had an higher effect than α,β-meATP and A317491 was able only to partially inhibit the effect of ATP and to completely decrease the effect of α,β-meATP. These differences are probably due to the presence of P2Y receptors, involved in PGE_2 release (Burnstock, 2004), which can be activated by ATP but not by α,β-meATP. The results of this paper

confirm ATP as an important extracellular signal involved in cartilage metabolism and indicate that the stimulation of purinergic pathways, mediated by P2X receptors, can increase the inflammation process associated to cartilage pathological conditions. On the other hand, studies concerning physiological mechanical stimulation on cartilage suggest that ATP, acting via purinergic pathways, may have beneficial effects on cartilage by stimulating proteoglycan synthesis and inhibiting NO production (Graff et al., 2000; Kono et al., 2006; Milward-Sadler et al., 2000, 2004; Chowdhury and Knight, 2006). In addition, it is reported that a chronic treatment of human chondrocytes with 100-500 μM ATP induced the loss of glycosaminoglycans but a single dose of 500 μM ATP increase proteoglycan and collagen synthesis (Picher et al., 2003). The reasons for this apparent discrepancy concerning the role of ATP and purinergic pathways are difficult to establish due to the limited knowledge concerning the expression, affinities and functional roles of purinergic receptors in cartilage. It is possible to hypothesize that various ATP levels associated to the cartilage physiological and/or pathological conditions can lead to the activation of different purinergic receptors subtypes. In conclusion, for the first time, the present study states in a quantitative way the presence and the binding parameters of $P2X_1$ and $P2X_3$ purinergic receptors in bovine chondrocytes. Binding and functional data on NO production and PGE_2 release confirm a pivotal role of these purinergic receptor subtypes in the inflammatory pathologies linked to the articular cartilage. Novel purinergic ligands could be potential targets for drug development in the future in several cartilage pathological conditions.

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CHAPTER 6:

$P2X_1$ and $P2X_3$ purinergic receptors differentially modulate the inflammatory response in human osteoarthritic fibroblasts-like synoviocytes.

INTRODUCTION

Fibroblast-like synoviocytes 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 fibroblast-like synoviocytes are detrimental to articular cartilage in different joint diseases such as OA and RA (Abramson et al., 2006; Varani et al., 2008a). 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 (Burnstock, 2006; Sawynok, 2007). 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 (Koles et al., 2007; Burnstock, 2008). 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 (Hoebertz et al., 2003; Picher et al., 2003). The pharmacological characterization of P2 receptors and the effects of nucleotides on fibroblast-like synoviocytes have not been investigated in detail although it has been shown that these cells respond to extracellular ATP (Hoebertz et al., 2003). Synergistic interaction between IL-6, PGE_2 and the presence of purinergic receptors in fibroblast-like synoviocytes may be important in the modulation of the joint tissue destruction including the damage related to inflammatory pathologies (Loredo and Benton, 1998; Caporali et al., 2008). It is well reported that ATP was able to mediate an increase of IL-6 and TNF-α in different cell lines (Inoue et al., 2007; Bulanova et al., 2009). The study of the pharmacologic modulation of the NF-kB pathways linked to p50 and p65 polypeptides suggest its involvement in the pathogenesis of several inflammatory diseases (Roman-Blas and Jimenez, 2006). 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 (Burnstock, 2006). The availability of [^{3}H]αβmeATP as radioligand to study P2X purinoceptors represent a useful tool in determining the distribution of these

receptors (Michel et al., 1995). 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 (Varani et al., 2008b,c). The aim of this study was to investigate the presence of P2X receptors in primary cultures of fibroblast-like synoviocytes from patients with OA and in SW 982 cells derived from human synovial sarcoma by using mRNA and western blotting assays. 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-kB 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, was obtained from NEN-Perkin Elmer Life and Analytical Sciences (USA). ATP, ADP, αβmeATP, BzATP, TNP-ATP, PPADS, A317491, suramin, vimentin, CD14 and von-Willebrand antibodies were purchased from Sigma-Aldrich Advanced Sciences (St. Louis, MO, USA). NF023, NF279, 2meSATP 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-kB 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 (North, 2002). Fibroblast-like synoviocytes were obtained by enzymatic digestion of synovial tissues derived from 27 patients with end-stage OA undergoing total joint replacement surgery (Miyashita et al., 2004; Bilgen et al., 2007). 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 fibroblast-like synoviocytes obtained from American Type Culture Collection (Bethesda, MD) (Christensen et al., 2005). To avoid the degradation of nucleotides, cells were incubated in serum-free medium during the pharmacological treatment with purinergic agonists and/or antagonists investigated.

Fibroblast-like synoviocytes 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 fibroblast-like synoviocytes, as previously described (Varani et al., 2008b; Miyashita et al., 2004). Human fibroblast-like synoviocytes and SW 982 were maintained in culture in DMEM, 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM). Human fibroblast-like synoviocytes 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 (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.

RT-PCR experiments

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method (Gessi et al., 2004). 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 fibroblast-like synoviocytes 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 sodium orthovanadate 1 mM, 4-(2-aminoethyl)-benzenesulfonyl fluoride 104 mM, aprotinin 0.08 mM, leupeptin 2 mM, bestatin 4 mM, pepstatin A 1.5 mM, E-64 1.4 mM [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 (Kiehntopf et al., 1995).

Saturation and competition binding assays

Saturation and competition binding assays were carried out in primary cultures of fibroblast-like synoviocytes 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 (Varani et al., 2008b,c). 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 $\Delta G°$ ($\Delta G° = -RTlnK_A$) which can be separated in its enthalpic and entropic contributions according to the Gibbs equation: $\Delta G° = \Delta H° - T\Delta S°$ (Borea et al., 2000).

NF-kB activation in human fibroblast-like synoviocytes

The NF-kB activation in nuclear extracts from human fibroblast-like synoviocytes and SW 982 cells was evaluated by detecting phosphorylated p65 and p50 proteins in nuclear extracts using the TransAM NF-kB kit (Active Motif, Carlsbad, USA) (Gomez et al., 2006). Phosphorylated NF-kB subunits specifically bind to the immobilized oligonucleotides containing the NF-kB consensus site (5'-GGGACTTTCC-3'). The primary antibody used to detect NF-kB 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 (Forrest et al., 2005; De Mattei et al., 2007).

Data and statistical analysis

The protein concentration was determined according to a Bio-Rad method with bovine albumin as reference standard (Bradford, 1976). A weighted non linear

least-squares curve fitting program Ligand was used for computer analysis of saturation and competition binding experiments (Munson and Rodbard, 1980). Functional experiments were calculated by non linear regression analysis using the equation for a sigmoid concentration-response curve (GraphPAD Prism, San Diego, CA, USA). 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 fibroblast-like synoviocytes

Fibroblast-like synoviocytes isolated from synovial of OA patients showed to be a homogenous population as demonstrated by their fibroblast-like morphology. Primary cultures of fibroblast-like synoviocytes (Figure 6.1A) and SW 982 cells (Figure 6.1B) also showed the expression of vimentin, a specific cellular marker for mesenchymal cells and fibroblast-like synoviocytes. 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 $P2X_{1\text{-}7}$ mRNA was shown and high levels of $P2X_1$, $P2X_3$ and $P2X_7$ mRNA were found in human fibroblast-like synoviocytes and SW 982 cells (Figure 6.2A). 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 (Figure 6.2B). Densitometric analysis of the bands obtained was also performed as shown in figure 6.2C,D.

Saturation and competition binding experiments

Saturation binding experiments in primary cultures of human fibroblast-like synoviocytes (Figure 6.3A,B) and in SW 982 (Figure 6.3C,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 fibroblast-like synoviocytes demonstrated the presence of an high affinity binding site (K_D= 3.1 ± 0.3 nM) and a receptor density (Bmax= 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) (Figure 6.3B,D). Affinity values obtained in [^{3}H]αβmeATP competition binding experiments by using selected purinergic agonists and antagonists in human fibroblast-like synoviocytes membranes are shown in table 6.1A. In human fibroblast-like synoviocytes membranes affinity values of the purinergic ligands used in functional experiments were obtained incubating 13 different concentration of purinergic agonists (Figure 6.4A) or antagonists (Figure 6.4B). 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 $P2X_3$ 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 (Figure 6.4C) and antagonists (Figure 6.4D). In human fibroblast-like synoviocytes 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 6.1B. 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 6.1B).

Thermodynamic analysis

The van't Hoff plots for purinergic ligands examined were essentially linear in the range 5-30°C in human fibroblast-like synoviocytes (Figure 6.5A,B) and in SW 982 cells (Figure 6.5C,D). Thermodynamic parameters (ΔG°, ΔH°, ΔS°) were reported in Table 6.2. 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 ΔS° = (ΔH°-

ΔG°)/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 fibroblast-like synoviocytes membranes carried out at 0, 10, 15, 20, 25 and 30°C in a thermostatic bath assuring a temperature of ± 0.1°C. The 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 of the selected purinergic compounds revealed that the binding of agonists was enthalpy and entropy driven (Table 6.2). 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 6.2).

Transcription factors in human fibroblast-like synoviocytes

NF-kB levels were evaluated studying P50 and P65 subunits activation. In human fibroblast-like synoviocytes, αβmeATP ($P2X_1$ and $P2X_3$ agonist) and BzATP ($P2X_7$ agonist) were able to increase of 85% and 152% the basal level of P65 subunit, respectively. βγmeATP ($P2X_1$ agonist) reduced of 58% the basal level of P65 subunit (Figure 6.6A). The capability of typical purinergic antagonists were investigated using A317491 and NF 023 which are able to block 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 present in human fibroblast-like synoviocytes 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 (Figure 6.7).

TNF-α, IL-6 and PGE_2 production

In both OA fibroblast-like synoviocytes 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 (Figure 6.6B), in IL-6 (Figure 6.8A) and PGE_2 production (Figure 6.8B). 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 (Figure 6.8A,B). In human fibroblast-like synoviocytes, 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 of 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.

Table 6.1A: Affinities, expressed as Ki or K_H and K_L values (nM) of selected purinergic agonists and antagonists in human fibroblast-like synoviocytes at different temperatures.

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
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

Table 6.1B: Affinities, expressed as Ki or K_H and K_L values (nM) of selected purinergic agonists and antagonists in SW 982 cells at different temperatures.

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
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

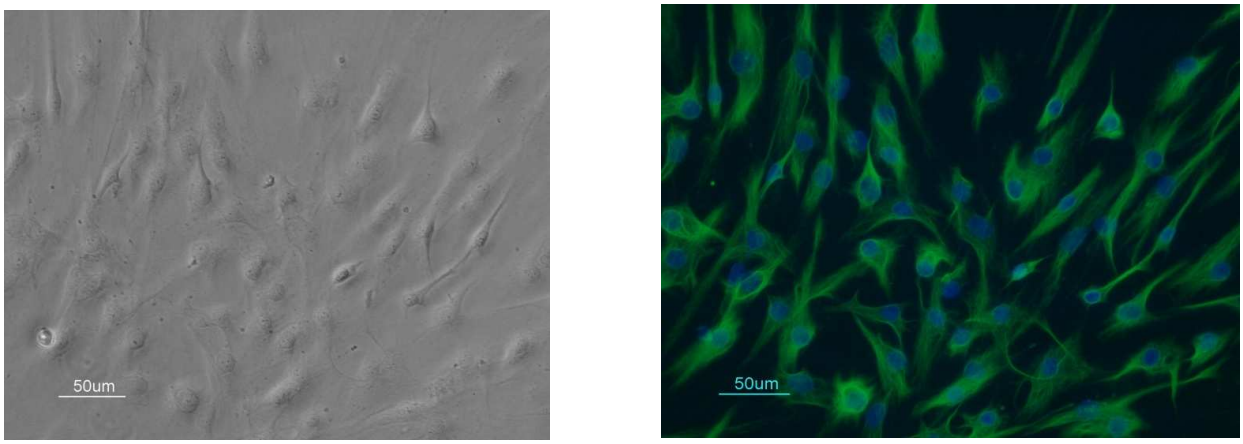
Table 6.2: Thermodynamic parameters for the binding equilibrium of [^{3}H]αβmeATP in human fibroblast-like synoviocytes (A) and SW982 cells (B) of selected purinergic agonists and antagonists.

(A) Ligand	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol/K)
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) Ligand	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol/K)
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

Figure 6.1: Culture of human fibroblast-like synoviocytes (A) and SW 982 cells (B). Left panels: phase contrast. Right panels: vimentin expression by immunofluorescence. Nuclei were counterstained in blue with DAPI.

A



B

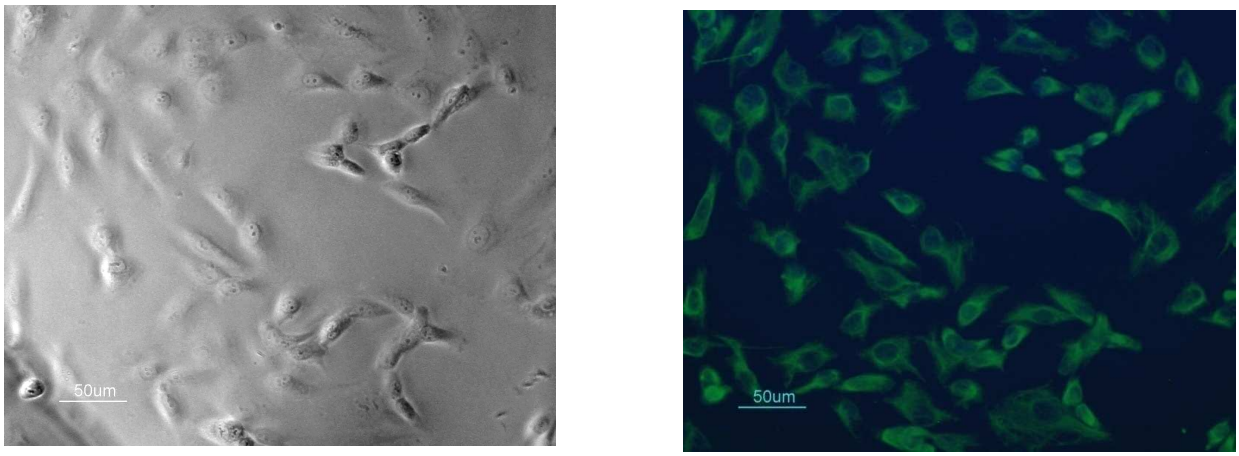


Figure 6.2: mRNA expression of P2X purinergic receptors (A) and western blotting analysis (B) in human fibroblast-like synoviocytes (SFs) and SW 982 cells in comparison with HEK293-hP2X$_1$ and HEK293-hP2X$_3$ cells. Densitometric analysis for hP2X$_1$ and hP2X$_3$ purinergic receptors are shown (C,D).

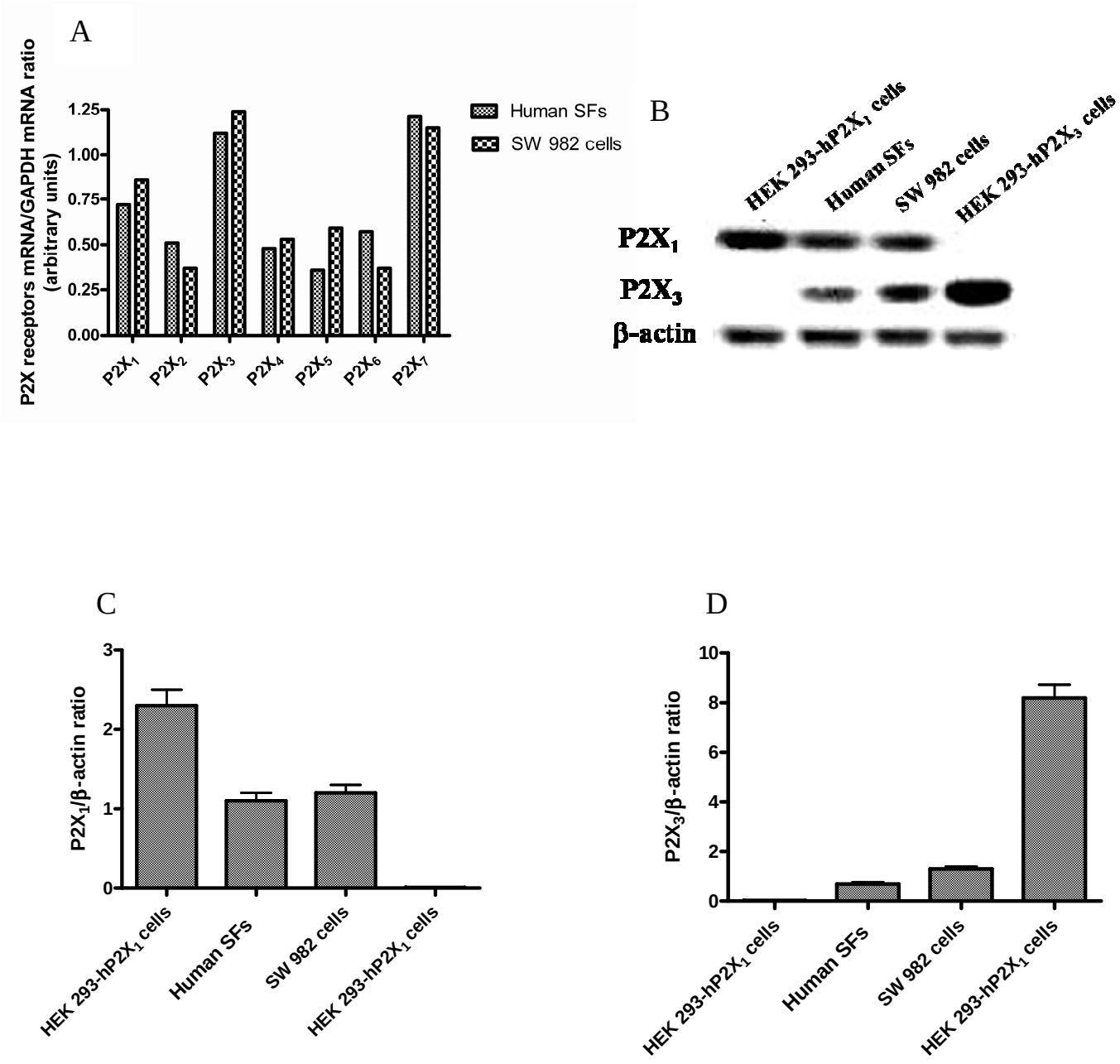


Figure 6.3. Saturation curves and Scatchard plot of [^{3}H]αβmeATP binding to P2X purinergic receptors in human fibroblast-like synoviocytes (A,B) and SW 982 (C,D) membranes at 5°C and 25°C.

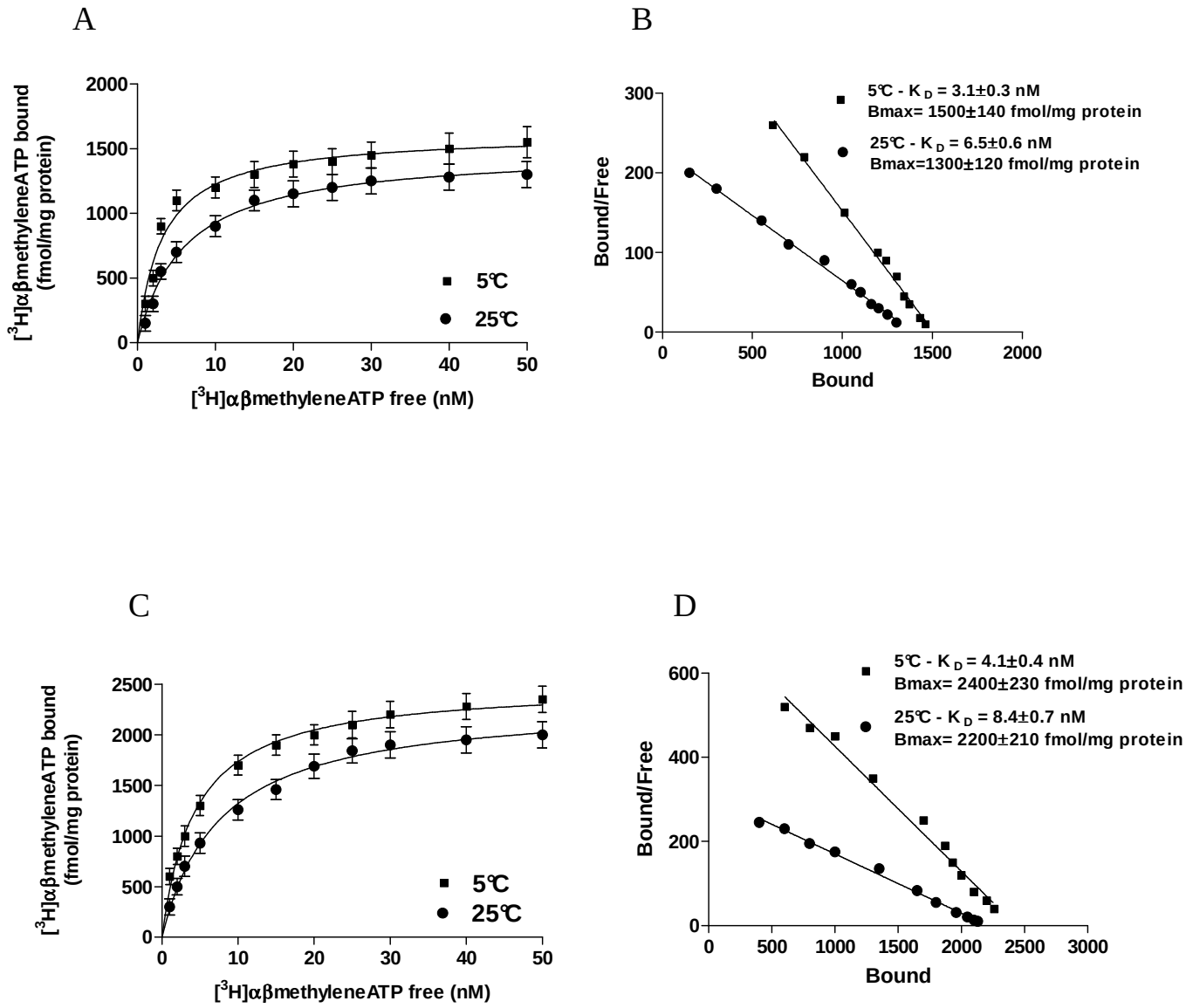


Figure 6.4: Competition curves of typical purinergic agonists and antagonists in human fibroblast-like synoviocytes (A,B) and in SW 982 (C,D) membranes.

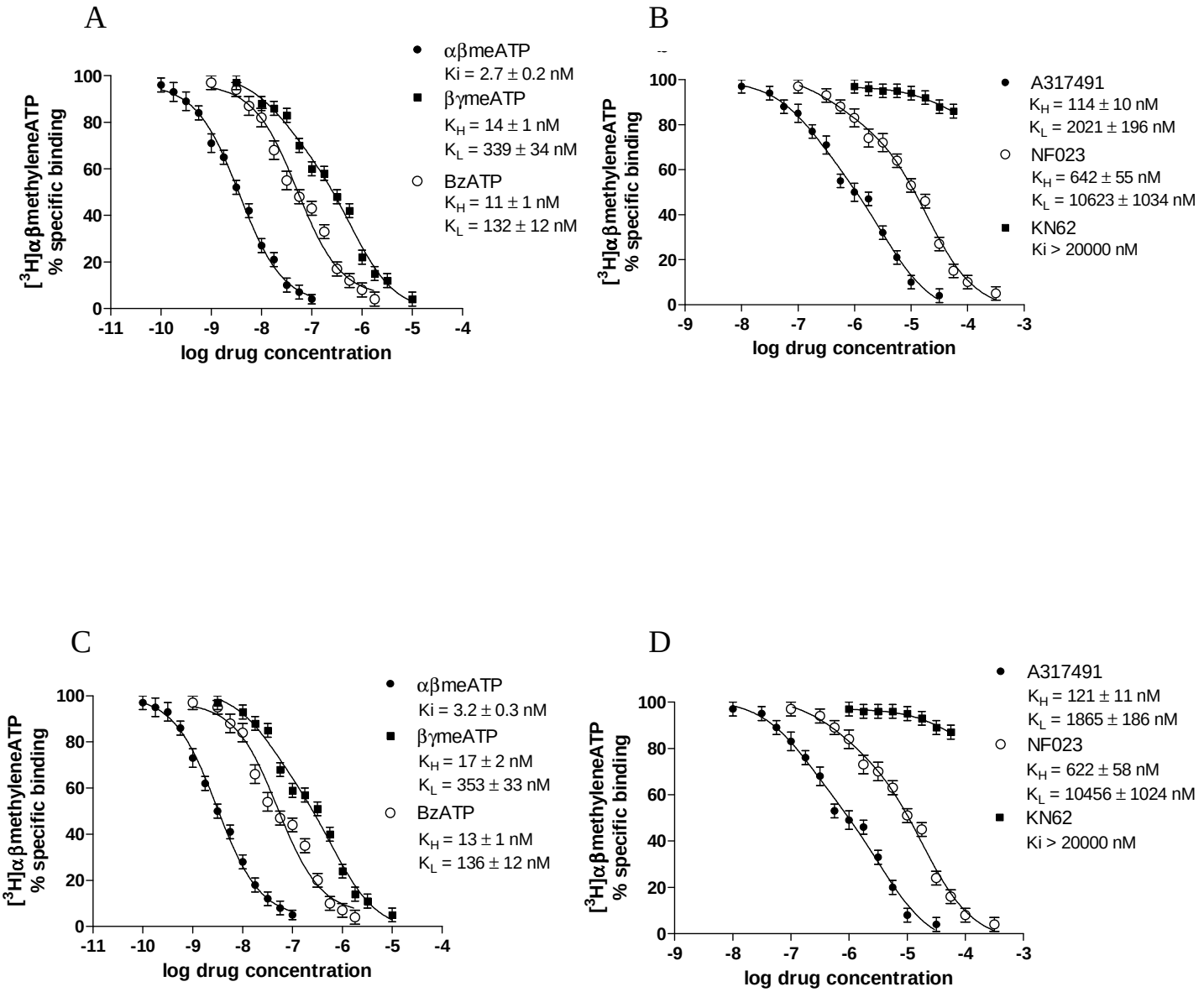


Figure 6.5. Van't Hoff plots in human fibroblast-like synoviocytes (A,B) and in SW 982 (C,D) membranes showing the effect of temperature on the equilibrium binding association constant, K_A, for P2X purinergic agonists (A,C) and antagonists (B,D).

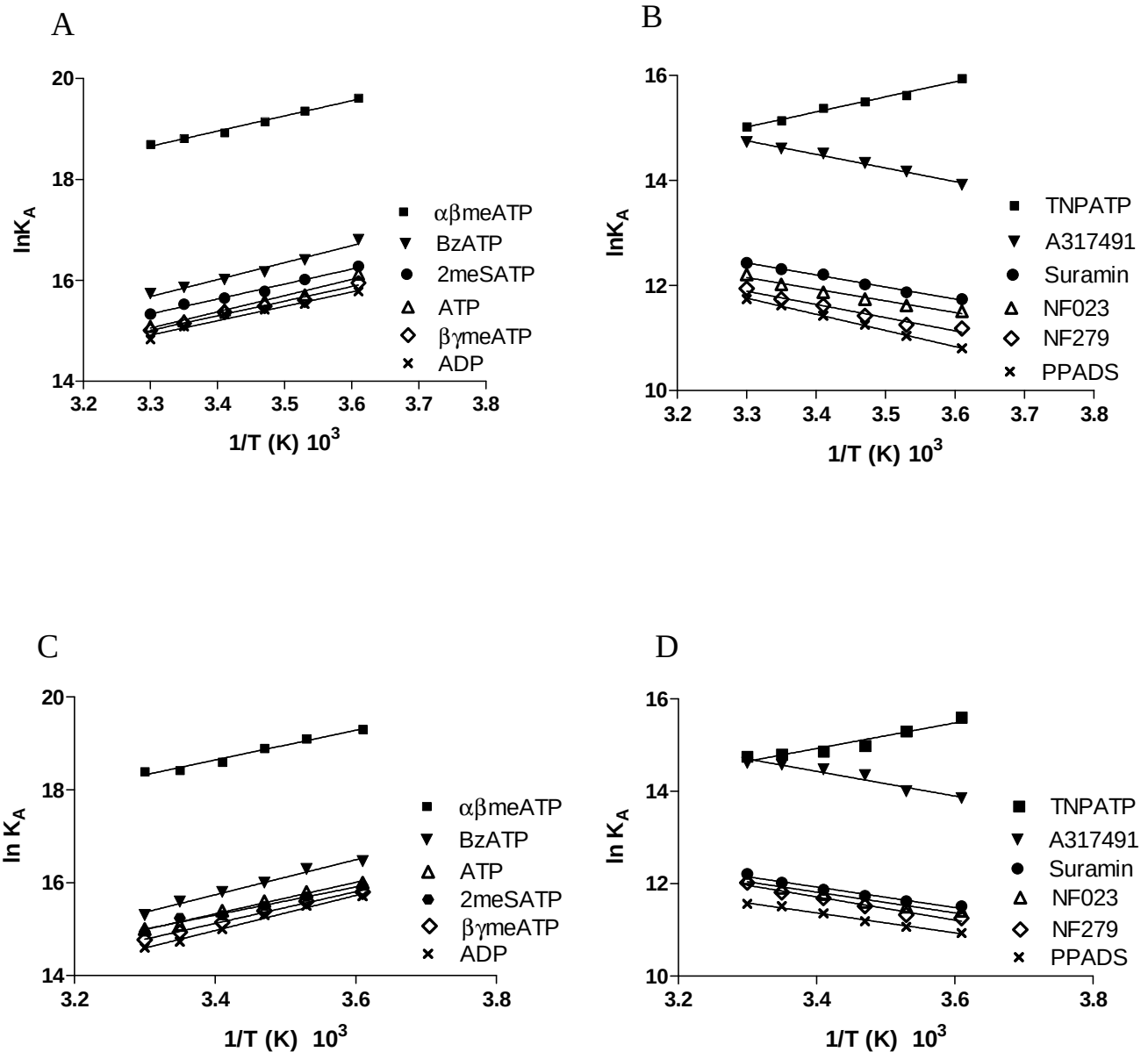
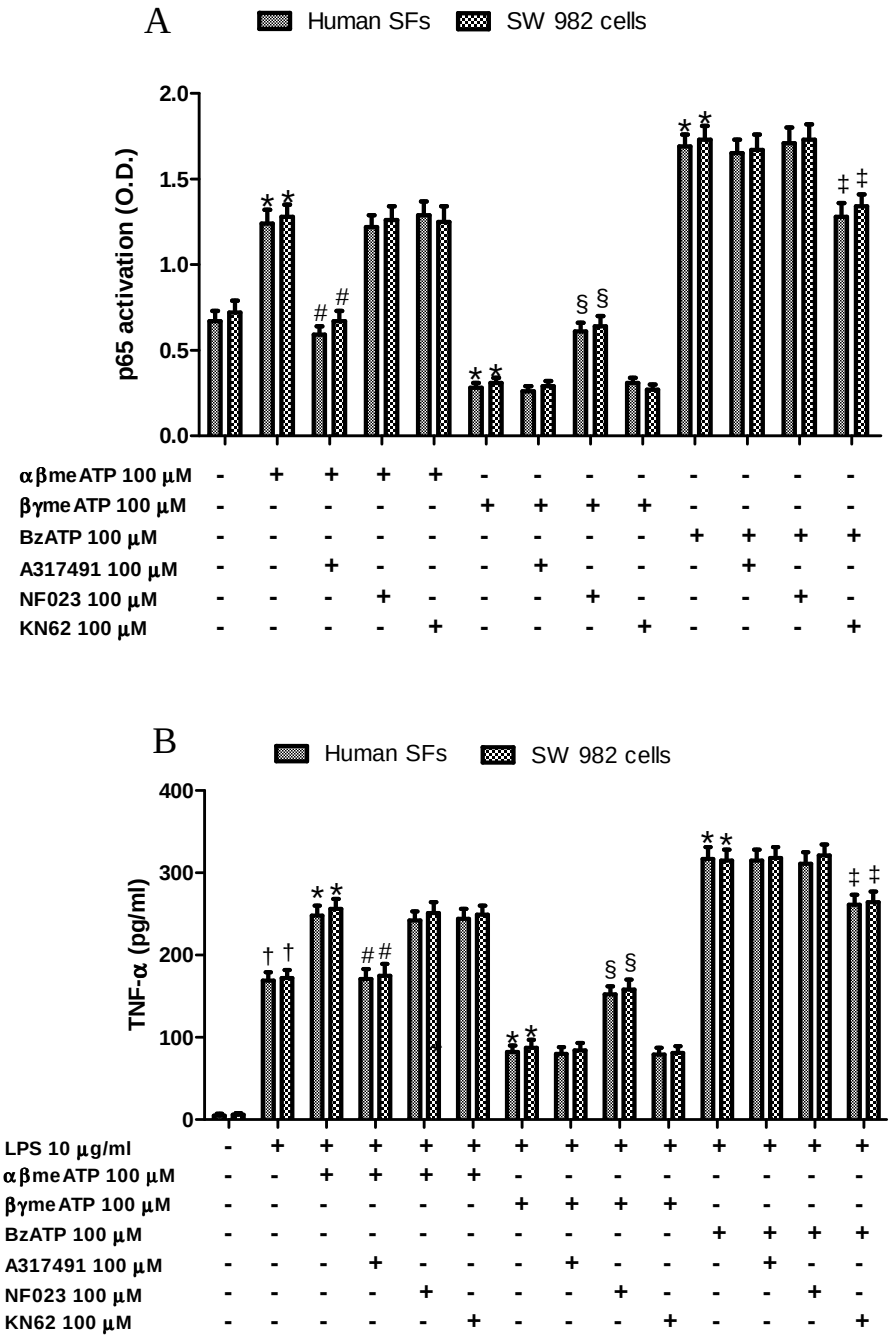
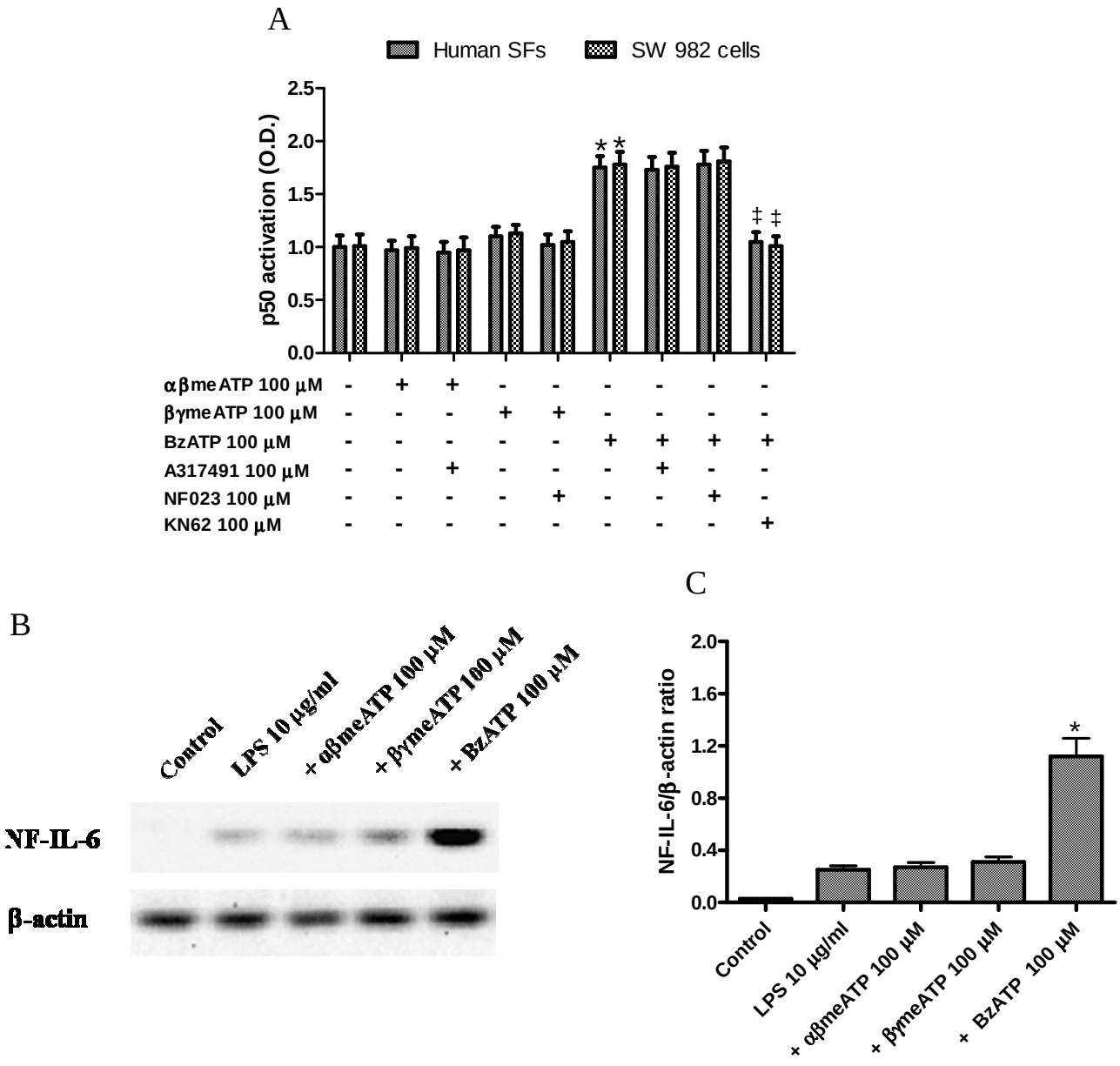


Figure 6.6: Effect of αβmeATP, βγmeATP and BzATP (100 µM) in human fibroblast-like synoviocytes (SFs) and in SW 982 cells on NF-kB activation by detecting phosphorylated p65 (A) in nuclear extracts and on TNF-α (B) levels in control conditions and stimulated by LPS (10 µg/ml).



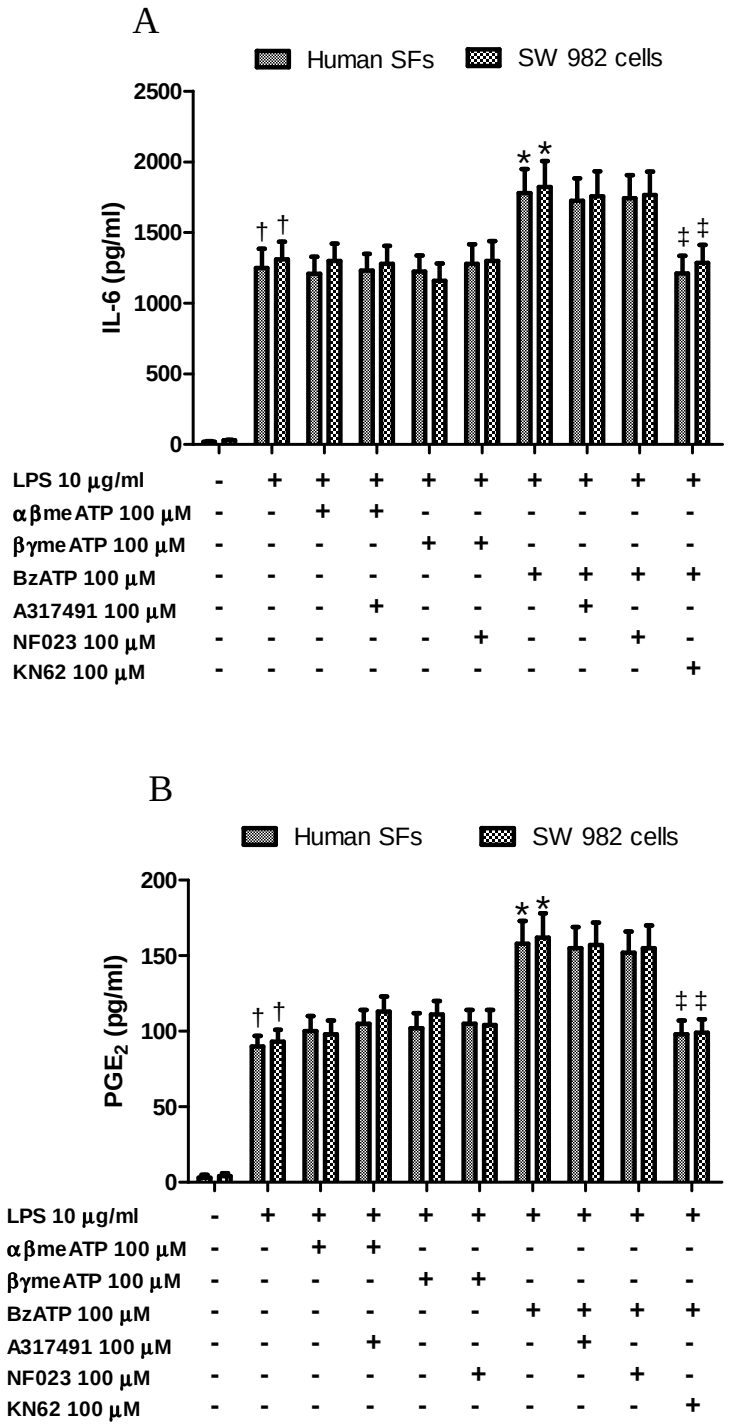
(A): *, p<0.01 vs control; #, p<0.01 vs αβmeATP; §, p<0.01 vs βγmeATP; ‡, p<0.02 vs BzATP; (B): †, p<0.01 vs control; *, p<0.01 vs LPS; #, p<0.01 vs αβmeATP; §, p<0.01 vs βγmeATP; ‡, p<0.02 vs BzATP.

Figure 6.7: Effect of αβmeATP, βγmeATP and BzATP (100 µM) in human fibroblast-like synoviocyes and in SW 982 cells on NFkB activation which was evaluated by detecting phosphorylated p50 (A) in nuclear extracts. No effect was present in western blotting analysis by αβmeATP and βγmeATP (100 µM) in human fibroblast-like synoviocytes 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)



(A): *, p<0.01 vs control; ‡, p<0.01 vs BzATP; (C): *, p<0.01 vs control

Figure 6.8: Effect of αβmeATP, βγmeATP and BzATP (100 µM) in human fibroblast-like synoviocytes (SFs) and in SW 982 cells on IL-6 (A) and PGE_2 (B) production. The effect of typical purinergic antagonists were also evaluated



†, $p<0.01$ vs control; *, $p<0.01$ vs LPS; ‡, $p<0.01$ vs BzATP

DISCUSSION

Nowadays no specific therapy, based on intracellular pathways of chondrocytes and/or fibroblast-like synoviocytes, 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 (Blom et al., 2007). 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 fibroblast-like synoviocytes 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 (Korkusuz et al., 2005; Goldring and Goldring, 2007). In this paper we report for the first time a binding and functional characterization of P2X purinergic receptors in human fibroblast-like synoviocytes. No works are present in the literature showing the presence and the role of $P2X_1$ and $P2X_3$ purinergic receptors in human fibroblast-like synoviocytes. In human fibroblast-like synoviocytes 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 fibroblast-like synoviocytes 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 (Varani et al., 2008b). 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 fibroblast-like 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 $\Delta H°$ and $\Delta S°$ values are independent of temperature (Varani et al., 2008b, c). 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 (Borea et al., 2000, 2004; Gilli et al., 2005). We previously reported that $P2X_3$ purinergic receptors are thermodynamically discriminated whilst $P2X_1$ receptor subtypes are not discriminated (Varani et al., 2008b). 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 fibroblast-like synoviocytes 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 (Varani et al., 2008b). These experimental data in human fibroblast-like synoviocytes 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 fibroblast-like synoviocytes. To this aim, the effect on NF-kB 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-kB activation and TNF-α release. These data are in agreement with those reported in literature regarding NF-kB that is one of the most important signalling pathways able to regulate pro-inflammatory cytokines such as TNF-α (Hong et al., 2008). 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 (Caporali et al., 2008). These results on IL-6 and PGE_2 production demonstrated a direct link in human fibroblast-like synoviocytes of different nuclear transcription factors involved in the modulation of pro-inflammatory molecules. In fact, NF-kB-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 (LeClair et al., 199; Uematsu et al., 2002). Finally, binding, thermodynamic and functional data demonstrated that $P2X_1$ and $P2X_3$ receptors present in human fibroblast-like synoviocytes 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 fibroblast-like synoviocytes 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 fibroblast-like synoviocytes suggesting their potential beneficial target for the treatment of inflammatory joint diseases.

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GENERAL CONCLUSION

Chronic inflammation is a significant factor in the pathophysiology of many forms of joint disease such as OA and RA. Chondrocytes and fibroblast-like synoviocytes are the two kind of cells particular involved in these diseases. In OA and RA there is a net loss of cartilage because the rate of catabolism exceeds the rate of synthesis and deposition of the macromolecular components of the extracellular matrix. Several chondrocyte activities are associated to cartilage damage, and include the up-regulation of NO and lipid inflammatory mediators such as PGE_2, as well as the increased production of matrix-degradating enzymes. As well as chondrocytes, fibroblast-like synoviocytes play a central role in the pathogenesis of joint destruction primarly by the secretion of a wide range of proinflammatory mediators including cytokines, growth factors and lipid mediators of inflammation. Growing evidence suggest that extracellular nucleotides, such as ATP and adenosine, might play important roles in the regulation of inflammatory processes, including the inflammatory process in joint diseases. In the first part of this study we analized the presence and the binding parameters of A_1, A_{2A}, A_{2B} and A_3 ARs in bovine chondrocytes and fibroblast-like synoviocytes. We found that all the adenosine receptors are present in these substrates and similarly distributed in both cell types showing high-affinity values in the nanomolar range and a receptor density from 30 to 83 fmol/mg of protein. We have also evaluated the capability of typical A_{2A} or A_3 adenosine agonists and antagonists to modulate cAMP levels. These compounds showed potency values in the nanomolar range, in agreement with their affinity in binding experiments, the antagonists were able to prevent the effect of cAMP induced by the agonists through a selective modulation of the adenylyl cyclase via A_{2A} or A_3 ARs. In addition, the levels of cAMP increased in response to NECA, a non selective agonist, suggesting that in physiological condition (presence of endogenous adenosine) the activation of A_{2A} may prevail over the A_3 mediated inhibitory action. We have also analized the role of adenosine analogs on PGE_2 release and COX-2 expression in bovine fibroblast-like synoviocytes. We have used as adenosine agonists CHA, CGS21680, NECA and Cl-IB-MECA. In fibroblast-like synoviocytes treated with two different type of inflammatory stimuli (TNF-α and LPS) we observed the increase of PGE_2 release. All the adenosine agonists, except

Cl-IB-MECA significally inhibited PGE_2 production. These data indicated for the involvement of A_1 and A_{2A} ARs in negative modulation of PGE_2 synthesis. We have also found that the changes in PGE_2 levels were associated to modification of COX-2 expression the primary enzyme controlling PGE_2 synthesis in response to inflammatory stimuli. Finally, we have performed a pharmacological and biochemical characterization of A_1, A_{2A}, A_{2B} and A_3 ARs in human fibroblast-like synoviocytes. We found that the ARs were present, well known adenosine agonists and antagonists showed affinity values in the nanomolar range and were coupled to stimulation or inhibition of adenylyl cyclase. A_{2A} and A_3 ARs activation inhibited p38 MAPK and NF-κB pathway, an effect abolished by selected adenosine antagonists. A_{2A} and A_3 agonists were able to diminish TNF-α and IL-8 production. A_1 and A_{2B} ARs were not implicated in the inflammation downstream whereas the stimulation of A_{2A} and A_3 ARs were closely associated with a down-regulation of the inflammatory status. These results indicate that A_{2A} and A_3 ARs could represent a potential target in therapeutic anti-inflammatory joint interventions. In the second part of the study the role of ATP in bovine chondrocytes and fibroblast-like synoviocytes have been evaluated. First of all thermodynamic parameters of the binding equilibrium of well-known purinergic agonists and antagonists were determinate in human $P2X_1$ and $P2X_3$ receptors expressed in HEK-293 cells. Our analysis showed that $P2X_1$ receptors are not thermodynamically discriminated and that the binding of agonists and antagonists was both enthalpy and entropy-driven. $P2X_3$ receptors were thermodynamically discriminated and purinergic agonist binding was enthalpy and entropy-driven, while antagonist binding was totally entropy-driven. As a consequence a biochemical and functional study on $P2X_1$ and $P2X_3$ receptors in bovine chondrocytes have been performed. We found that bovine chondrocytes expressed $P2X_1$ and $P2X_3$ purinergic receptors and thermodynamic parameters indicated that purinergic binding is enthalpy- and entropy-driven for agonists and totally entropy-driven for antagonists. Purinergic agonists such as ATP and αβ-meATP were able to increase NO and PGE_2 release. A purinergic antagonist, A317491, was able to block the stimulatory effect on functional experiments mediated by the agonists, suggesting the potential role of novel purinergic antagonists in the treatment of pathophysiological disease involved in inflammatory of the joints. In addition, the pharmacological characterization of $P2X_1$ and $P2X_3$ purinergic

receptors in fibroblast-like synoviocytes obtained from OA patients and in SW982 cells derived from human synovial sarcoma have been performed. Moreover their possible involvement in modulating the inflammatory response by assessing the activation of NF-kB and the production of TNF -α, IL-6 and PGE_2 have been investigated. Interestingly $P2X_1$ and $P2X_3$ receptors were present in human fibroblast-like synoviocytes and SW982 cells. In these cells purinergic agonists and antagonists have a different thermodynamic behavior. This study report, for the first time the presence of $P2X_1$ and $P2X_3$ purinergic receptors in human fibroblast-like synoviocytes. Moreover from the functional point of view $P2X_1$ receptors exhibit anti-inflammatory effects, by reducing the activation of NF-kB and the release of TNF-α, whereas $P2X_3$ receptors mediate an opposite response suggesting pro-inflammatory effects.

In conclusion, overall these data demonstrate the presence of A_1, A_{2A}, A_{2B}, A_3 ARs and $P2X_1$, $P2X_3$ purinergic receptors in bovine chondrocytes and fibroblast-like synoviocytes. The functionality of these purine receptors suggest the use of selective adenosine and/or purinergic ligands in the treatment of inflammatory diseases. In addition, the expression and the signal transduction system of adenosine and purine receptors in human fibroblast-like synoviocytes highlight the potential pharmacological use of novel purine ligands in disorders associated with inflammation.

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Varani K, Surprenant A, Vincenzi F, Tosi A, Gessi S, Merighi S, Borea PA (2008) Binding thermodynamic characterization of human $P2X_1$ and $P2X_3$ purinergic receptors. Biochem Pharmacol 75(5):1198-1208.

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MEETINGS

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