UNIVERSITÀ DEGLI STUDI DI MILANO

Facoltà di Farmacia
Dipartimento di Scienze Farmacologiche

Corso di Dottorato di Ricerca in Scienze Farmacotossicologiche, Farmacognostiche e Biotecnologie Farmacologiche (XXIII CICLO)

Graduate School in Pharmacological Sciences / Scuola di Dottorato in Scienze farmacologiche

TESI DI DOTTORATO DI RICERCA

DIFFERENTIAL ROLE OF DISTINCT P2 RECEPTOR SUBTYPES IN CARDIOMYOCYTE DEATH INDUCED BY ISCHEMIC/HYPOXIC STRESS

BIO/14

Tesi di dottorato di:
SIMONA COSENTINO
MATRICOLA: R07554

TUTOR: Chiar.ma Prof.ssa Maria Pia ABBRACCHIO

COORDINATORE: Chiar.mo Prof. Guido FRANCESCHINI

ANNO ACCADEMICO 2009/2010

INDEX

1 INTRODUCTION

1.1 PURINERGIC TRANSMISSION

Purines and pyrimidines have widespread and specific extracellular signalling actions in the regulation of a variety of biologic functions in many tissues. For many years, the focus of interest in purines and pyrimidines has been the involvement of ATP in cell metabolism and its role as an energy source. The concept that ATP is an extracellular signalling molecule took a long time to be accepted (Abbracchio and Burnstock, 1998). With the development of new research tools, such as molecular cloning, purinergic signalling has become an established principle not only in the rapid signalling involved in neurotransmission, but also in a wide range of other biological processes, including release of cytokines and hormones (Firestein et al., 1996; Rasi et al., 1996), regulation of cell proliferation, differentiation and apoptosis in tissues as diverse as the skin, skeletal muscle, bone, nervous and immune system (Abbracchio et al., 1996). Thus, alterations in purinergic signalling may contribute to the development of several diseases, in particular disorders of the immune system, inflammation, cardiac ischemia, neurodegeneration, osteoporosis, cancer and many others. On this basis, the understanding of the precise role of purinergic signalling in various organs and systems may help the identification of novel targets for the development of more effective therapeutic approaches to currently incurable human diseases.

1.1.1 Purines and pyrimidines

It has been demonstrated that, following mechanical stress, non secretory tissues can also release nucleotides (Lazarowski et al., 2000) that may signal to the same secretory cell (autocrine stimulation) as well as to adjacent cells (paracrine stimulation). Following injury, extracellular adenine (purine) and uracil (pyrimidines) nucleotides can be released as a consequence of cell lysis. In these circumstances, ATP can reach millimolar concentrations in the extracellular milieu, although in physiological conditions relatively low concentrations of the nucleotide can induce rapid and specific responses. Like adenine nucleotides, uracil nucleotides regulate a broad range of functions: nervous cell excitation, muscle cell proliferation, endothelial adhesion, leukocyte chemotaxis, spermatogenesis, hormones and histamine release, acid balance in epithelial intestinal cells, mucociliary clearance in airway epithelium, and superoxide production. Furthermore, basal release of UTP from human platelets has been described (Lazarowski and Harden, 1999), suggesting a role for this nucleotide in endothelial and muscular cell stimulation during thrombosis. More recently, during myocardial infarction, human venous plasma levels of UTP have been also demonstrated to be released (Wihlborg et al., 2006) and in *vivo* experiments in the rat have shown that UTP reduces infarct size and improves myocardial function (Shainberg et al., 2009). Once released in the extracellular environment, nucleotides are rapidly degraded by ubiquitous ecto-nucleotidases (Zimmermann, 2000), a family of phosphatases expressed on the cell surface that are able to metabolize different nucleotides. ATP

hydrolysis produces ADP, AMP and adenosine, whether UTP is degraded to UDP, UMP and uridine. All these metabolites can function as extracellular signalling molecules, thus tissue response to nucleotide release is the result of the effects of the nucleotide and of its degradation products on target cells. Distinct classes of ecto-nucleotidases with different properties and different substrate specificities have been identified so far (Zimmermann, 1999). Some are membrane proteins, but soluble forms released in the extracellular milieu following a proteolytic process have also been described. Ecto-nucleotidases can also be released with ATP from sympathetic nerve terminals and they represent one of the mechanisms used to turn off neurotransmitter signalling (Todorov et al., 1997). There is a significant increase in ectonucleotidase activity (NTPdaseI) in the hearts of patients with ischaemic heart disease (Kittel et al., 2005) that could represent a compensatory mechanism against increased nucleotide levels during chronic ischaemia.

1.1.2 Purinergic receptors

Based on the actions of purine nucleotides in a wide variety of tissue, in 1978, Burnstock proposed some criteria for the classification of purinergic receptors in two families: the P1 receptors, selectively activated by adenosine and the P2 receptors, responding to ATP and ADP. The distinction between P1 and P2 receptors has been officially approved and has been adopted by the International Union of Pharmacology (IUPHAR) Subcommittee for the Nomenclature and Classification of Purinoceptor and is now universally used (Abbracchio and

Burnstock, 1994; Fredholm et al., 1997). Four members of the P1 family of G protein-coupled receptors have been identified (A1, A2A, A2B and A3; (Fredholm et al., 1994) and 15 members of the P2 family have been cloned so far (Abbracchio et al., 2006; Khakh and Henderson, 2000). A further classification was suggested for P2 receptors on the basis of their pharmacological profiles (Burnstock, 2006), and on their vascular effects (some induce vasoconstriction whereas others mediate an opposite effect). Further studies have led to a better structural characterization of the P2 receptors and to the distinction between ionotropic purinergic receptors mediating rapid effects and metabotropic P2 receptors with a later onset of response (Dubyak, 1991). In 1994, together with the cloning of the first members of the family, a new classification of P2 receptors was proposed (Abbracchio and Burnstock, 1994). Two main groups were identified, the P2X family of ligand-activated channels and the G protein-coupled P2Y receptors. This new classification allows to compare purinergic transmission to acetylcholine, glutamate, serotonin and GABA systems, which are known to involve both metabotropic and ionotropic receptors (Abbracchio and Burnstock, 1998). Up to date, at least 7 P2X subtypes ($P2X_{1\text{-}7}$) and 8 P2Y members ($P2Y_{1,2,4,6,11,12,13,14}$) have been cloned from different animal species (Abbracchio et al., 2006). The missing numbers in the P2Y series correspond to receptors cloned from vertebrates different from mammals and for which no mammalian orthologs have been identified so far, or to receptors that have not yet been functionally characterized. Recently, a new putative member of the P2Y receptor family, previously known as the “orphan” receptor

GPR17, has been identified and has been showed to be specifically activated by both uracil nucleotides (UDP, UDP-glucose and UDP-galactose) and cysteinyl-leukotrienes (cysLTs) (Ciana et al., 2006).

1.1.2.1 P2X receptors

P2X receptors are membrane ion channels that contain intrinsic pores that switch conformation from closed to open on binding ATP, allowing ions to flow. The flow of ions is a key step in signalling, because it changes the trasmembrane potential as well as local ion concentrations (Khakh and North, 2006). ATP can elicit rapid responses (<10ms) via these ion channels, resulting in selective permeability to Na+, K+, and Ca_2+ cations (North, 2002). In vertebrates, seven genes encode P2X receptor subunits, which are 40–50% identical in amino acid sequence. The seven P2X subunits range from 379 ($P2X_6$) to 595 ($P2X_7$) amino acids in length. Each subunit has two hydrophobic, putative membrane-spanning segments (TM1 and TM2) of sufficient length to cross the plasma membrane, separated by an ectodomain that contains 10 conserved cysteine residues which form disulfide bonds to give the correct conformation to the receptor (Clyne et al., 2002; Ennion and Evans, 2002; Vial et al., 2004). The ectodomain (about 300 residues) is thought to form an extracellular loop containing the ATP binding site and sites for antagonists and modulators (Khakh et al., 2001). The NH_2 and COOH termini are intracellular (Newbolt et al., 1998; Stoop et al., 1999; Torres et al., 1998) and comprise the cytosolic domain. The C-terminal regions diverge in sequence considerably. This topology is the simplest

among ionotropic receptors (Figure 1.1). The amino acid identity between P2X receptor subunits is distributed throughout the extracellular domain. All the P2X receptor subunits have consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr), and some glycosylation is essential for trafficking to the cell surface. The extracellular domain carries few conserved glycine (G) and proline (P) residues which are involved in conformational changes subsequent to the ligand-receptor binding. Extracellular protons, bivalent cations and some metals are P2X receptors allosteric modulators. In addition, P2X receptors can be modulated via the phosphorylation of serine (S) and threonine (T) residues. Functional expression studies have highlighted the existence of heteromeric, $P2X_1/_5$, $P2X_2/_3$, $P2X_2/_6$, $P2X_4/_6$, $P2X_4/_7$ and $P2X_5$ receptors which assemble with any others, except $P2X_7$ (Guo et al., 2007; North, 2002). With the use of pharmacology and gene knock-out/down approaches, it has also become clear that P2X receptors are involved in a wide and growing range of pathophysiological processes (Khakh and North, 2006).

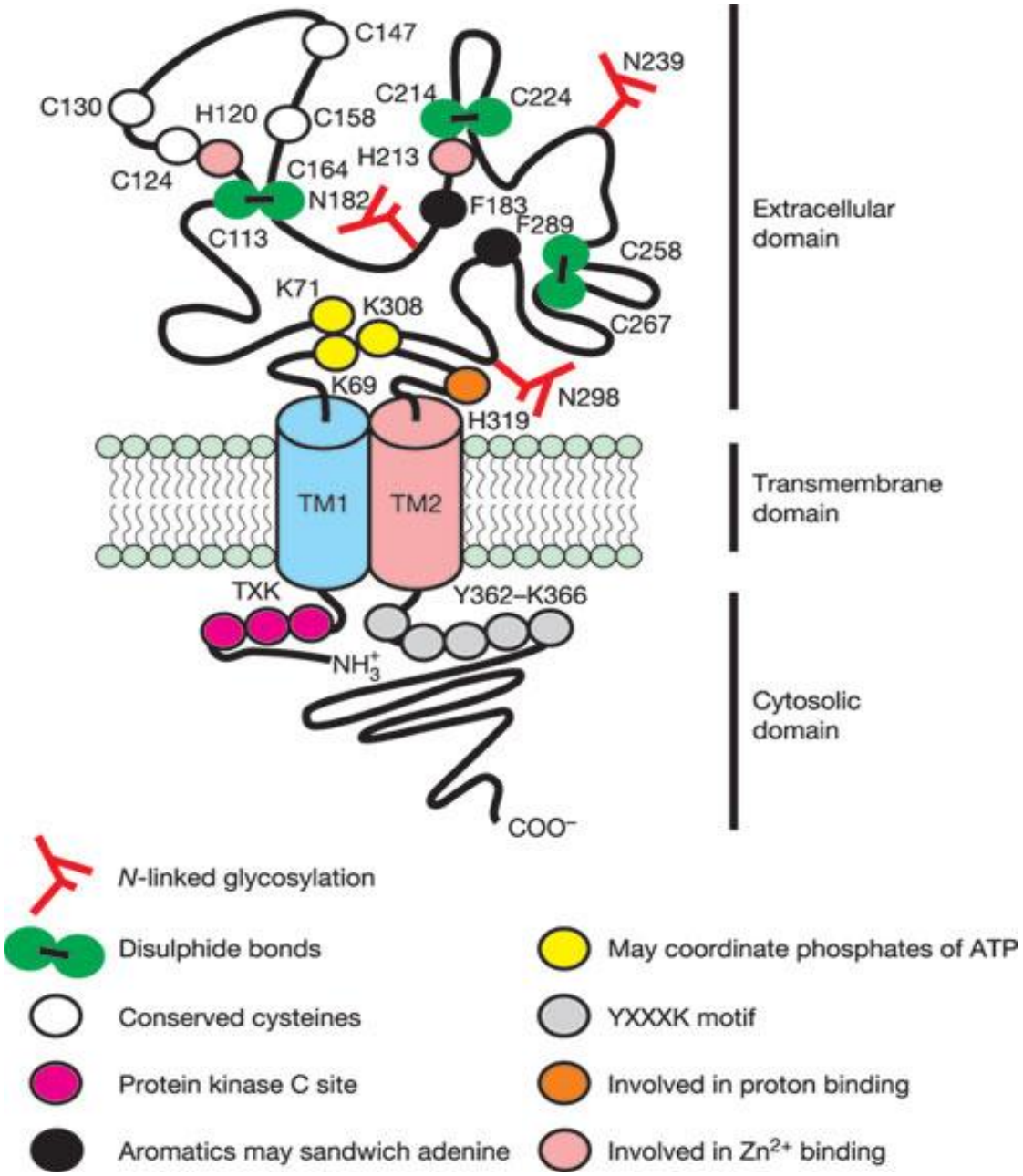


Figure 1.1: Schematic representation of a P2X receptor.

An individual subunit showing the two transmembrane structure and amino acid residues that are implicated in ligand binding and that maintain the conformation of the subunit (modified from Khakh et al., 2006)

1.1.2.2 P2Y receptors

P2Y receptors belong to the superfamily g protein-coupled receptors (GPCR). GPCRs are a family of membrane receptors responding to a wide variety of ligands such as nucleotides, biogenic amines, peptides and other small molecules (Marchese et al., 1999). The binding of the GPCR to its specific ligand results in the activation of the associated heterotrimeric (α, β and γ subunits) G protein that can mediate a number of intracellular responses. In particular, ligand binding to its receptor results in a decreased affinity of the α subunit of the G protein for GDP, that is thus released and substituted for by GTP. This binding causes a conformational modification in the G protein and the dissociation of the α subunit from the βγ complex. Both α and βγ subunits can activate signal transduction pathways (Rebois et al., 1997). The receptor proteins of the P2Y receptor subtypes contain the typical features of GPCRs including 7 predicted hydrophobic transmembrane regions (TMs) connected by 3 extracellular loops (ELs) and 3 intracellular loops. The proteins of the human receptors consist of 328 ($P2Y_6$) to 377 ($P2Y_4$) amino acids corresponding to a predicted molecular mass of 41-53 kDa of the glycosylated proteins. The biochemical analysis of the P2Y-receptor proteins has shown that at the level of the cell membrane are modified by N-linked glycosilation (Erb et al., 1995). All know P2Y-receptor subtypes possess at their extracellular domains 4 cysteine residues, which are likely to form 2 disulfide bridges. The P2Y receptor-family shows a relatively high diversity in the amino acid composition to P2X receptors and in contrast, P2Y receptor genes do not contain introns in the coding sequence,

except for the $P2Y_{11}$ receptor. Site-directed mutagenesis of the $P2Y_1$ and $P2Y_2$ receptors has shown that some positively charged residues in transmembrane domains 3, 6 and 7, which are localized close to intracellular loops, are crucial for receptor activation by nucleotides (Erb et al., 1995; Jiang et al., 1997). They probably interact with the negative charges of the phosphate groups of nucleotides. Actually, the eight P2Y receptors identified so far have a H-x-x-R/K motif in TM6 that might be important for agonist activity (Figure 1.2).

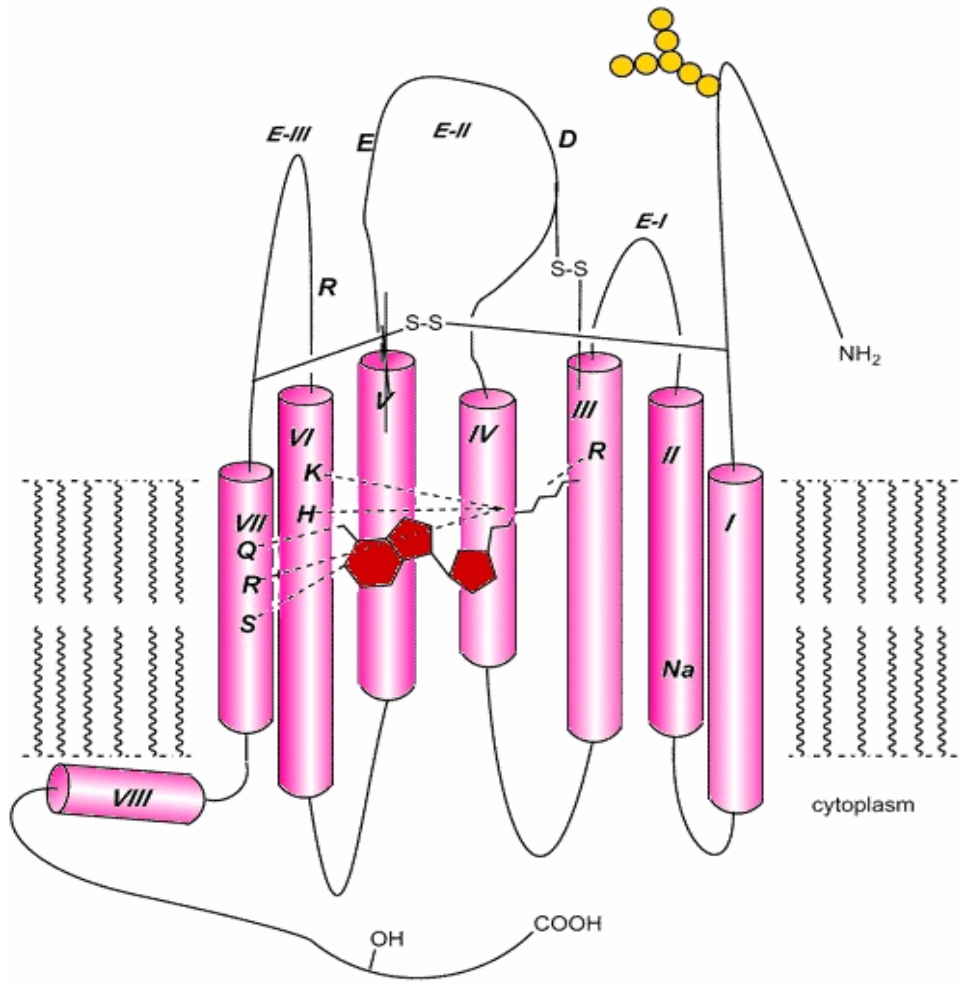


Figure 1.2: Schematic representation of a P2Y receptor showing the seven transmembrane structure and amino acidic residues that are implicated in ligand binding.

Model shows features of the hP2Y$_1$ receptor important for nucleotide binding both within the TMs (3, 6, and 7) and ELs (2 and 3), four Cys residues (-S), which are conserved among P2Y subtypes, form disulfide bridges. The location of the putative glycosylation site is conserved within the P2Y family. Modulatory residues for the activation of the P2Y$_1$ receptor are also shown. TM = transmembrane; EL= extracellular loop (modified from Jacobson et al., 2002).

From a phylogenetical point of view, the eight human P2Y receptors can be subdivided into two distinct subgroups characterized by a relatively high level of sequence divergence. The first subgroup encompasses $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$, whereas the second subgroup encompasses $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Abbracchio et al., 2006). Receptors in the first subgroup also share a YQ/ K-x-x-R motif in TM7 (proposed to participate in ligand binding), whereas in receptors of the second subgroup another motif (K-E-x-x-L) which might affect ligand binding characteristics is found (Abbracchio et al., 2006). These two P2Y receptor subgroups also differ in their primary coupling to transductional G proteins. In particular, receptors in the first subgroup (i.e., $P2Y_{1,2,4,6,11}$) all principally use G_q/G_{11} to activate PLCβ/IP3 pathway and release intracellular calcium, whereas receptors in the second subgroup (i.e., $P2Y_{12,13,14}$) almost exclusively use the $G_{i/o}$ class of G proteins to lower cAMP levels. Secondary couplings have been also reported, especially for receptors of the first subgroup in heterologous expression systems (Kottgen et al., 2003; Simon et al., 2002; White et al., 2003). Among receptors of the second group, $P2Y_{13}$ has been also reported to couple to $G\alpha_{16}$ and stimulate phospholipase C in recombinant systems overexpressing this G protein (Communi et al., 2001; Marteau et al., 2003);(Fumagalli et al., 2004). Such "promiscuity" of G protein coupling may depend on the indirect activation of additional G protein subtypes within protein complexes containing the P2Y receptor.

1.1.2.3 Pharmacological features of P2Y receptors

There is increasing interest in the expression of P2Y receptors in pathological conditions and in the therapeutic potential of P2Y receptor related compounds. Both $P2Y_1$ and $P2Y_{12}$ receptors costitute targets for antithrombotic therapy; progress in exploring structure-activity relationships has been achieved for these receptors and to a lesser extent for the $P2Y_2$ receptor. However, most of the P2Y receptor subtypes are still lacking potent and selective synthetic agonists and antagonists (Abbracchio et al., 2006).

1.1.2.3.1 ADP- preferring receptors: $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$

ADP is the natural agonist at the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors, and it interacts at these subtypes with generally greater affinity than does ATP (Marteau et al., 2003; Palmer et al., 1998). At $P2Y_1$ receptors, derivatives of adenosine 5′-diphosphate tend to be full agonists, while ATP appears to be a partial agonist. At $P2Y_{12}$ receptors, adenosine 5′-diphosphate derivatives act as agonists, and 5′-triphosphate derivatives act as antagonists (Gachet, 2005). At $P2Y_{13}$ receptors, both ADP and ATP appear to be full agonists; at the $P2Y_1$ receptor, 2-MeSADP has been used widely for activation, but this compound also activates the $P2Y_{12}$ and $P2Y_{13}$ receptors. Notably, the 20-deoxy N6-methyl derivative MRS2179 is a protypical selective $P2Y_1$ antagonist. The favored ribose-ring conformation for each of the subtypes of the $P2Y_1$-like family has been established using conformationally restricted ribose equivalents. The later generation of synthetic bisphosphate antagonists incorporates a rigid substitute for the normally flexible

ribose ring. MRS2279 and MRS2500 are generally useful as selective, high-affinity antagonists of the $P2Y_1$ receptor in various species (Kim et al., 2003). The (N)-methanocarba ring in these nucleotide analogs both enhances receptor affinity and improves stability toward nucleotidases. Antagonists of the $P2Y_1$ receptor of moderate affinity might also be derived from acyclic nucleotides (bisphosphates and bisphosphonates), such as MRS2496 (Costanzi et al., 2007). The screening of structurally diverse chemical libraries by the pharmaceutical industry has led to non-nucleotide antagonists of the $P2Y_1$ receptor (Pfefferkorn et al., 2008). A tetrahydro-4-quinolinamine derivative inhibited the $P2Y_1$ receptor effects and platelet aggregation (Morales-Ramos et al., 2008). Extensive structure-activity studies of ATP derivatives as antagonists of the platelet $P2Y_{12}$ receptor resulted in high-affinity selective antagonists of interest as antithrombotic agents. The thienopyridines, such as clopidogrel, were serendipitously identified as inhibitors of platelet aggregation by ADP 20 years before the cloning and identification of their target, the $P2Y_{12}$ receptor. They act as liver-activated prodrugs, the active metabolites of which are irreversible inhibitors of the $P2Y_{12}$ receptor. A drug development program by AstraZeneca to design $P2Y_{12}$ receptor antagonists has introduced numerous directly acting $P2Y_{12}$ receptor antagonists. The observation that ATP acts as an antagonist at this ADP-activated subtype has enabled the introduction of various 50-triphosphate analogs as selective receptors probes, and one of them, AR-C69931MX 42 (Cangrelor), has been tested clinically as an antithrombotic agent. One of the products of this effort is ticagrelor 43 (AZD6140), an

uncharged nucleoside derivative with a high affinity at the $P2Y_{12}$ receptor, which is currently in clinical trials (Springthorpe et al., 2007; Tantry et al., 2007)(Wallentin, 2009). The search for selective non-nucleotide antagonists of the $P2Y_{12}$ receptor, for potential use as antithrombotic agents, is continuing. The agonist potency at the $P2Y_{13}$ receptor is ADP > 2-MeSADP > ATP. A selective $P2Y_{13}$ receptor antagonist MRS2211 is a derivative of PPADS, a nonselective P2Y and P2X receptor antagonist derived from pyridoxal phosphate (Kim et al., 2005). MRS2211 has the disadvantage of containing a phosphate ester group and an aryl diazo linkage, both of which are subject to instability in tissue systems. The nonselective P2X/P2Y antagonist Reactive blue 2 (RB2) and its derivatives are known to block action at $P2Y_1$ receptors, however high potency and selectivity have not been achieved (Brown and Brown, 2002; Jacobson et al., 2002). The polysulfonate suramin and its derivatives, in addition to displaying trypanocidal drug properties, are relatively nonselective P2 antagonist with, in general, reversibility upon washout. Within the P2Y family, suramin has been characterized as an antagonist of $P2Y_2$ receptors (Wildman et al., 2003). Derivatives of PPADS have also been shown to antagonize $P2Y_1$ receptor effects in a competitive fashion, although at micromolar concentrations (Lambrecht et al., 2002). Most of the ligands of P2Y receptors identified so far are polyanionic molecules that do not readily cross the cell membrane. This raises a major problem of bioavailability, this lack of systemic action can be an asset in case of topical applications, such as spray or eye drops. The hydrolysis of nucleotide compounds by ectonucleotidases constitutes another

limiting factor partially overcome by the development of more stable dinucleotide compounds. Orally active uncharged antagonists (e.g. thienopyridines and ticagrelor) are available only for the $P2Y_{12}$ receptor.

1.1.2.3.2 ATP preferring receptors: $P2Y_{11}$

ATP is the preferred native ligand at $P2Y_{11}$ receptors (Communi et al., 1999), and ATP-γ-S is a more potent agonist than ATP. The $P2Y_{12}$ antagonist AR-C69931MX 42 (cangrelor) acts as a potent agonist at the $P2Y_{11}$ receptor (Boeynaems et al., 2005). Selective antagonists of the $P2Y_{11}$ receptors are still unknown. $P2Y_{11}$ is not expressed in rodents.

1.1.2.3.3 Selective receptor for UDP: $P2Y_6$

Uridine 5′-diphosphate derivatives activate the $P2Y_6$ receptor more potently than the corresponding 5′-triphosphates (Malmsjo et al., 2000; Muller, 2002); thus, UDP is a selective agonist at this subtype. The β-thiodiphosphate was shown to be more potent than UDP in activation of the $P2Y_6$ receptor and more stable to degradation. Various diisothiocyante derivatives were found to be potent antagonist of human $P2Y_6$ as well as other P2Y receptors (Mamedova et al., 2004). A 1,4-di-(phenylthioureido) butane derivative (MRS2578) selectively inhibited UDP-induced PLC activity through both human and rat $P2Y_6$ receptors expressed in 1321N1 human astrocytes and was inactive at human $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors.

1.1.2.3.4 Receptors with mixed selectivity: $P2Y_2$ and $P2Y_4$

The $P2Y_2$ receptor is activated nearly equipotently by UTP and ATP but is not activated by the corresponding 5′-diphosphates, i.e., UDP and ADP. The $P2Y_4$ receptor is primarily activated by uracil nucleotides, depending on the species. In the rat, ATP is also a $P2Y_4$ agonist, but in humans it acts as a $P2Y_4$ antagonist. Uridine β-thiodiphosphate (UDP- β-S) and the γ-thiophosphate (UTP-γ-S) are selective agonists for $P2Y_6$ and $P2Y_2$/$P2Y_4$ receptors, respectively (Malmsjo et al., 2000). Numerous substitutions of the uracil ring of UTP have been reported to reduce potency at the $P2Y_2$ receptor (Muller, 2002). The adenine dinucleotide Ap4A is a potent agonist at the rat $P2Y_4$ receptor and is less potent than ATP at the $P2Y_2$ receptor. Other uracil dinucleotides, such as INS365 (Up4U), also potently activate the $P2Y_2$ receptor (Shaver et al., 2005). The dependence of potency at various P2Y receptors on the number of bridging phosphate units in the dinucleotide series indicates an optimum at the tetraphosphate. Newer-generation $P2Y_2$ receptor agonists, such as INS37217 (Up4dC), have been reported (Pendergast et al., 2001; Yerxa et al., 2002). $P2Y_2$ receptor agonists are of clinical interest for the treatment of pulmonary and ophthalmic diseases and possibly cancer. Suramin is a weak antagonist at the $P2Y_2$ receptor with an IC_{50} of 48 μM (Muller, 2002). At concentration of 100 μM, reactive blue-2 effectively blocks rat $P2Y_4$ receptors, but only partially blocks human $P2Y_4$ receptors. Flavonoids have been identified as a new lead for the design of $P2Y_2$ receptor antagonists. AR-C118925, is reported to selectively antagonize $P2Y_2$ receptor (Jacobson and Boeynaems).

1.1.2.3.5 Receptor for sugar nucleotides: $P2Y_{14}$

The most recently cloned receptor, $P2Y_{14}$, responds to UDP-glucose and has a sequence more similar to the $P2Y_{12}$ and $P2Y_{13}$ receptors than to the other P2Y subtypes (Abbracchio et al., 2003). The $P2Y_{14}$ receptor is also activated by UDP-galactose, UDP-glucuronic acid and UDP-N acetylglucosamine (Chambers et al., 2000). Of these endogenous ligands, to date only UDP-glucose has been shown to be released extracellularly by a variety of cell lines (Lazarowski et al., 2003). At present, no selective antagonists are available, although it has to be underlined that the currently available P2 receptor antagonists have not been tested on this receptor.

1.1.2.3.6 New P2Y receptor for uracil nucleotides: GPR17

The recently deorphanized P2Y- like receptor GPR17 has been reported to respond to both uracil nucleotides and cysteinyl-leukotrienes, such as UDP-glucose and LTD_4 (Ciana et al., 2006). GPR17 is expressed in organs typically undergoing ischemic damage (i.e., brain, heart and kidney), thus representing a new pharmacological target for acute and chronic neurodegeneration.

1.2 HEART ISCHEMIC DISEASE

Cardiovascular diseases (CVD) have emerged as dominant chronic disease in western countries. In the 21st century, CVD are also predicted to become the main cause of disability and death worldwide. In Europe, every year CVD kills 2 million of people, 250.000 only in Italy. Ischemic heart disease refers to a lack of oxygen due to inadequate perfusion of the myocardium, which causes an imbalance between oxygen supply and demand. Myocardial ischemia occurs at virtually any age, but the frequency rises progressively with increasing age (especially men over 40 and women over 50) and with presence of important risk and lifestyle factors that predispose to atherosclerosis, such as hypertension, smoking, diabetes mellitus, obesity, genetic hypercholesterolemia, and other causes of hyperlipoproteinemia. The most common cause of myocardial ischemia is obstructive atherosclerosis disease of epicardial coronary arteries (Figure 1.3).

1.2.1 Atherosclerosis

Atherosclerosis is the main cause of ischaemic stroke and cardiovascular disease and is now considered to be an inflammatory disease (Hansson, 2005). The formation of a plaque starts with the accumulation of cholesterol followed by invasion of macrophages taking up cholesterol and becoming foam cells. The plaque can be stabilised by smooth muscle cell formation of a fibrous cap that covers the lipid-rich region. However, stimulation of inflammation by oxidised lowdensity lipoprotein activates macrophages and dendritic cells into antigen-presenting cells,

activating T lymphocytes, resulting in release of cytokines and metalloproteinases degrading the fibrous cap. The end result is a vulnerable plaque and, when it ruptures, its highly thrombogenic contents activates platelets and causes the formation of a local thrombus occluding the artery or embolising, resulting in ischaemic stroke or myocardial infarction. Upon occurrence of inadequate myocardial perfusion caused by coronary atheroslerosis, myocardial tissue oxygen tension falls and causes transient disturbances of mechanical, biochemical and electrical function of the myocardium. The abrupt development of severe ischemia is associated with almost instantaneous failure of normal muscle contraction and relaxation.

1.2.2 Ischemic diseases classification

When ischemia is transient, it may be associated with angina pectoris, when it is prolonged can lead to acute myocardial infarction (MI). Patients with ischemic heart disease fall into two large groups:

1- patients with stable angina secondary to chronic coronary artery disease;

2- patients with acute coronary syndromes (ACS). The latter is composed of:

a. patients with acute myocardial infarction (MI) with ST-segment elevation on their presenting electrocardiogram (STEMI)

b. patients with unstable angina (UA) and non ST-segment elevation MI (UA/NSTEMI).

a. <u>UA/NSTEMI</u> is associated to a reduction in oxygen supply and/or an increase in myocardial oxygen demand superimposed on a coronary obstruction. This condition is normally an aggravation of a stable coronary artery disease and requires medical or mechanical interventions.

b. <u>STEMI</u> generally occurs when coronary blood flow decreases abruptly after a thrombotic occlusion of a coronary artery previously affected by atherosclerosis. STEMI occurs when a coronary artery thrombus develops rapidly at a site of vascular injury. In most cases, infarction occurs when an atherosclerotic plaque fissures, ruptures, or ulcerates and when conditions favor thrombogenesis, so that a mural thrombus forms at the site of rupture and leads to coronary artery occlusion. The extension of myocardial damage and scar formation caused by coronary occlusion depends on:

1- the territory supplied by the affected vessel

2- whether the vessel becomes totally or partially occluded

3- the duration of coronary occlusion

4- blood quantity supplied by collateral vessels to the infarcted tissue

5- the myocardium oxygen demand whose blood supply has been suddenly limited

6- native factors that can produce early spontaneous lysis of the occlusive thrombus

7- the adequacy of myocardial perfusion in the infarct zone when flow is restored in the occluded epicardial coronary artery.

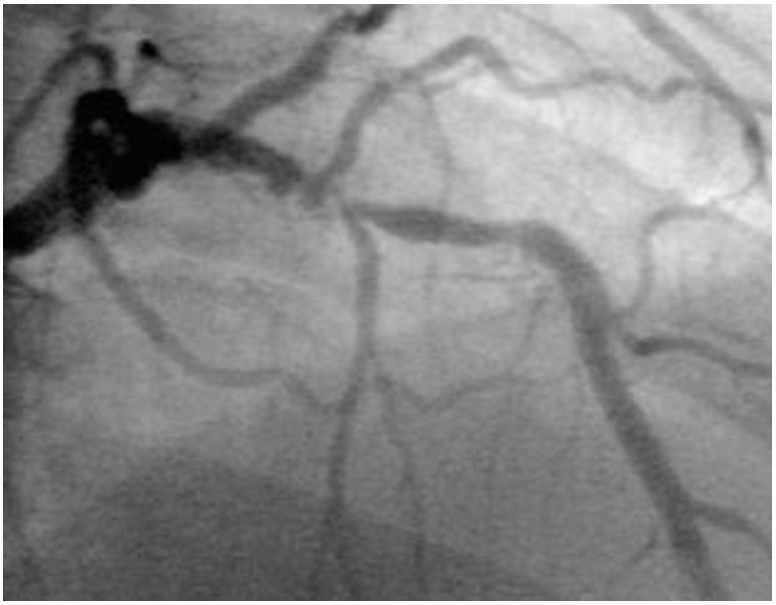


***Figure.1.3**: **Coronary angiogram.** It is possible to observe a severe proximal stenosis of the left descending coronary artery due to an atherosclerotic plaque.*

1.2.3 Injury in the pathogenesis of ischemic disease

Because oxygen delivery to the heart is closely coupled to coronary blood flow, a sudden arrest of regional perfusion following a thrombotic coronary occlusion quickly leads to the suspension of aerobic metabolism, depletion of creatine phosphate, and the onset of anaerobic glycolysis. This is followed by the accumulation of tissue lactate, a progressive reduction in tissue ATP levels, and a accumulation of catabolites. As ischemia continues, tissue acidosis develops and there is an efflux of potassium into the extracellular space. Subsequently, ATP levels fall below those required to maintain critical membrane function, resulting in the onset of myocyte death. The temporal evolution and extent of irreversible tissue injury is variable and dependent

on trasmural location, residual coronary flow, and the hemodynamic determinants of oxygen cosumption. Irreversible injury begins after 20 minutes of coronary occlusion in the absence of significant collaterals (Kloner and Jennings, 2001). Factors that increase myocardial oxygen consumption (e.g., tachycardia) or reduce oxygen delivery (e.g., anemia, arterial hypotension) accelerate the progression of irreversible injury. Reperfusion immediately causes myocytes necrosis and sarcolemmal disruption, with the leakage of cell contents into the extracellular space. The injury is further amplified by the reentry of leukocytes into the area of injury. At later time points, myocytes initially salvaged can undergo apoptosis, which can contribute to further delayed myocardial injury.

1.2.4 Myocyte cell death

Cell death arises from distinct mechanisms in myocardial infarction. It has been reported to have features of apoptosis, necrosis and autophagy.

1.2.4.1 Apoptosis

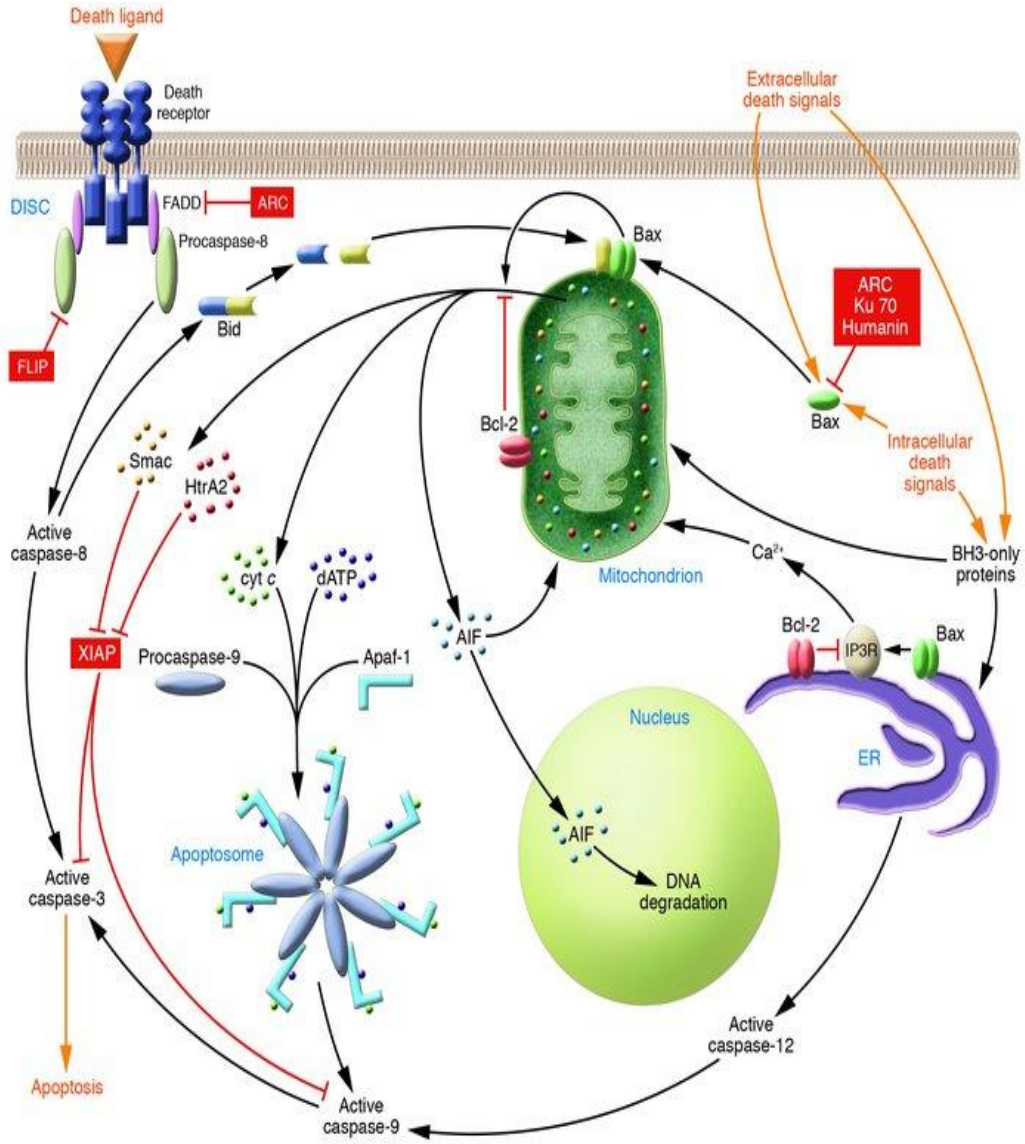
Apoptosis, or programmed cell death, is an evolutionary conserved process that allows multicellular organisms to selectively remove cells through a highly orchestrated program of cell suicide. The ensemble of proteins that are responsible for activating apoptosis is preprogrammed into the genetic code of the cells that are destined to die. However, under pathological circumstances such as acute ischemia, the apoptotic program can be triggered inappropriately, resulting in inadvertent cell death.

Apoptosis requires energy and activation of specific biochemical steps that are involved in triggering and executing cell death through activation of “intrinsic (mitochondrial) and “extrinsic” (death receptor mediated) pathway that both lead to the activation of executioner caspases (Figure 1.4). The extrinsic pathway is specialized to trasduce signals from soluble and cell-bound death ligands, which bind and activate their cell surface death receptors (Peter and Krammer, 2003). The more ancient intrinsic pathway is responsible for trasducing most apoptotic stimuli converging at the mitochondria to trigger the release of apoptogenic proteins and at endoplasmic reticulum (ER) to stimulate the release of luminal calcium (Scorrano et al., 2002). During apoptosis the cell shrinks and eventually breaks up into small, membrane-surronded fragments. The latter often contain bits of condensed chromatin, referred to as apoptotic bodies. Maintenance of plasma membrane integrity until late in the apoptotic process allows the dying cell to be engulfed by macrophages, which prevents the release of the reactive intracellular contents, thereby preventing an inflammatory reaction. Endonuclease-mediated breakdown of DNA in apoptosis leads to the generation of multiples of 180 to 200 base pair fragments. Programmed myocyte cell death is the prevailing form of myocardial damage produced by occlusion of a major epicardial coronary artery, whereas necrotic myocytes cell death follows apoptosis and contributes minimally to the progressive loss of myocytes after infarction (Anversa et al., 1998). The fact that apoptosis plays a role in the tissue damage seen after myocardial infarction has pathological and therapeutic implications. Because apoptosis is a highly regulated process, a better understanding of

the circumstances that specifically trigger apoptosis during and after myocardial infarction, and a better understanding of the cellular mechanism that control apoptosis, could lead to therapeutic strategies to limit the amount of tissue damage in patients with myocardial infarction.

1.2.4.2 Necrosis

Necrosis is usually defined in a negative fashion, as a type of cell demise that involves rupture of the plasma membrane without the hallmarks of apoptosis and without massive autophagic vacuolization. The principal features of necrosis include a gain in cell volume (oncosis) that finally culminates in rupture of the plasma membrane, and the unorganized dismantling of swollen organelles. Necrosis is considered to be harmful because it is often associated with pathological cell loss which results in an inflammatory response (Vakkila and Lotze, 2004). An inflammatory response is an important component of ischemia injury. An important distinction between necrosis and apoptosis is the manner in which DNA is degraded; in necrosis, DNA is broken down randomly sized fragments. A typical necrosis mechanism involves opening of mitochondrial permeability trasition pore (MPTP) in the inner mitochondrial membrane. Events during ischemia can contribute to MPTP opening, that results in loss of the eletrical potential difference due to the proton gradient that normally exists across the inner membrane, and in ATP depletion. Cyclophilin D is an important regulator of MPTP; cells lacking cyclophilin D are resistant to oxidative stress but sensitive to apoptosis stimuli (Whelan et al.).



F***igure 1.4: Intrinsic and extrinsic cell death pathways in cardiac myocytes.***

In the extrinsic pathway, ligand binding induces death receptors to recruit Fas-Associated protein with Death Domain (FADD), which recruits procaspase-8. Within this complex (DISC), procaspase-8 dimerizes and is activated. Caspase-8 activates procaspase-3, which cleaves cellular proteins causing cell death. In the intrinsic pathway, extracellular and intracellular stimuli signal to the mitochondrium through a variety of BH3-only proteins and through Bax (modified from Foo et al., 2005).

1.2.4.3 Autophagy

In contrast to necrosis and apoptosis, autophagy is primarly a survival mechanism. Authophagy is a slow, spatially restricted phenomenon in which organelles, proteins, and lipids are sequesteres within double-membraned vacuoles and finally digested by lysosomal hydrolases (Kroemer and Jaattela, 2005). This process provides cells with amino acids, free fatty acids, and energy in times of nutrient deprivation. There are studies showing that inhibition of autophagy during prolonged ischemia is detrimental, suggesting a potential protective role with its function in helping cells survive nutrient deprivation (Takagi et al., 2007).

1.2.5 Therapies for ischemic heart disease

The ideal therapy would have the following activities: it would minimize cardiomyocytes loss by reducing cell death, promote return of ischemic dysfunctional myocardium to normal function, stimulate revascularization of the ischemic region by enhancing angiogenesis, and regenerate viable tissue by replacing that lost as a consequence of the ischemic event, thereby preserving contractile function and reducing the opportunity for scarring. Current therapies address the process of heart ischemic failure by different approaches: in the acute setting reducing, the duration of the ischemia by surgically or pharmacologically removing the vascular blockage; in the chronic phase relieving infarcted heart work with drugs and devices (Fraser et al., 2004).

1.2.5.1 Limitation of the infarct size

The quantity of myocardium that becomes necrotic as a consequence of coronary artery occlusion is determined by factors outside the site of the occlusion. While the central zone of the infarct contains necrotic tissue that is irretrievably lost, the fate of the surrounding ischemic myocardium may be improved by the timely restoration of coronary perfusion, reduction of myocardial oxygen demand, and prevention of the accumulation of noxious metabolites. Up to one-third of patients with STEMI may achieve spontaneous reperfusion of the infarct-related coronary artery within 24h. Pharmacological treatments (i.e. by fibrinolysis) or mechanical revascularization with coronary angioplasty (PCI) accelerate the reperfusion through the occluded infarct-related artery in those patients in whom spontaneous thrombolysis would ultimately have occurred.

1.2.5.2 Pharmacologic therapy

Angiotensin-converting enzyme inhibitors (ACE-inhibitors) decrease afterload by antagonizing the vasopressor effect of angiotensin, thereby decreasing the amount of work that the heart must perform. It is also believed that angiotensin directly affects cardiac remodeling and blocking its activity can consequently reduce the deterioration of cardiac function. Diuretics therapy is indicated for relief of congestive symptoms. As cardiac frequency is the main determinant of myocardial oxygen comsuption, its reduction is an important protection strategy against myocardial ischemia. β-blockers reduce blood pressure and contractibility, and it has been shown they also reduce necrotic area after

cornonaric occlusion; the administration of β-blockers can decrease mortality and improve left ventricular function (Faxon, 2004).

1.2.5.3 Angiogenesis

Angiogenesis is the biologic process of forming new blood vessels. Under physiological conditions, angiogenesis is regulated by the local balance between endogenous stimulators and inhibitors of this process. Both acute and chronic myocardial ischemia have been clearly shown to stimulate angiogenesis in many experimental models. Therapeutic angiogenesis stimulates the growth of new blood vessels. Traditional coronary revascularization therapies such as coronary angioplasty or bypass graft surgery act by restoring blood flow through pre-existing coronary vessels. One limitation of these approaches may be the failure to normalize myocardial perfusion, due to the concomitant presence of small resistance vessel disease. In contrast, therapeutic angiogenesis is based on the concept that coronary collateral development may be stimulated by pharmacological or molecular means and can limit myocardial ischemia.

1.2.5.4 Surgical therapy

Surgery may have a role in ameliorating ischemic heart failure. Patients with dysfunctional but viable myocardium (a condition named hybernation) and diffuse coronaropathy benefit from coronary bypass surgery in terms of ventricular performance and prognosis. Further, a viable option for severe remodeling due to

large scars is ventricular cardiomyoplasty, although long-term benefits have been recently challenged.

1.3 P2 RECEPTORS IN THE CARDIOVASCULAR SYSTEM

In the cardiovascular system, evidence is accumulating to indicate that P2 receptors mediate important physiologic effects, including vasoconstriction and vasodilatation, growth of vascular smooth muscle and endothelial cells, angiogenesis, vascular remodelling, coagulation, platelet aggregation, inflammation and several aspects of cardiac function. The purinergic system and its receptors are also involved in development of myocardial infarction, heart failure and xenograft rejection. The physiological effects of the purinergic signalling system are dependent on the release of extracellular nucleotides, the degradation by ectonucleotides, the type of P2 receptors expressed on the cells, their desensitisation rates and their second messengers. P2 receptors have highly specific organ distributions and they can be rapidly up- or downregulated. Among the 15 receptor identified subtypes, one ($P2Y_{12}$) is the target for one of the most widely used medical drugs the platelet inhibitor Clopidogrel (Plavix) (Erlinge and Burnstock, 2008).

1.3.1 Regulation of vascular tone

Vasoconstriction produced by ATP released as a cotransmitter with noradrenaline (NA) from perivascular sympathetic nerves was recognised early (Burnstock, 1976). Later, it was shown that ATP acted on endothelial cells to release endothelial derived relaxing factor, such as nitric oxide (NO) resulting in vasodilatation (Burnstock, 1987). A dual purinergic neural and endothelial control of vascular tone was established. The most important contractile

receptors on the vascular smooth muscle cells (VSMC) are the ATP $P2X_1$ receptor, the ATP/UTP $P2Y_2$ receptor, the UDP $P2Y_6$ receptor, and the ADP $P2Y_{12}$ receptor

1.3.1.1 Endothelial regulation

Extracellular nucleotides produce several important effects mediated by activation of endothelial cells. Shear stress and hypoxia are important stimuli for both ATP and UTP release from endothelial cells (Burnstock, 1987). Both of them reduce forearmvascular resistance in a prostaglandin and NO independent way (Hrafnkelsdottir et al., 2001), indicating an important role for endothelium-derived hyperpolarising factor in P2 receptor-mediated vasodilatation in man. The $P2Y_1$ receptor seems to be of major importance in most vascular beds (Erlinge et al., 1998). $P2Y_2$ and to a lesser degree $P2Y_6$ are also important endothelial P2Y receptors (Wang et al., 2002). Extracellular ATP in the circulation is rapidly degraded into ADP, AMP and adenosine by ectonucleotidases. Vascular NTPDase1 (CD39) is an endothelial cell membrane protein with both ecto-ATPase and ecto-ADPase activities (Kaczmarek et al., 1996). Ectonucleotidases are also released by shear stress from endothelial cells (Yegutkin et al., 2000). Reactive hyperaemia is the massive increase in blood flow that starts when a blood vessel is opened after a period of ischaemia. It has been proposed that different purines may mediate three phases of the reactive hyperaemia (Olivecrona et al., 2004). According to this hypothesis, ATP would mediate the first part of the hyperaemia via endothelial $P2Y_2$ or $P2X_4$ receptors. ATP would then be degraded to ADP, which mediates peak hyperaemia via endothelial $P2Y_1$ receptors, followed by degradation

of ADP to adenosine resulting in late phase hyperaemia mediated via A2a receptors on smooth muscle cells.

1.3.1.2 Red blood cells as regulators of vascular tone

Blood flow increases in response to decreased tissue oxygen levels. Red blood cells (RBCs) contain millimolar amounts of ATP and possess the membrane-bound glycolytic enzymes necessary for its production (Bergfeld and Forrester, 1992). ATP is released in response to reductions in oxygen tension and pH (Ellsworth et al., 1995). It has been shown *in vitro* that vessels dilate in response to low O_2 levels only when blood vessels are perfused with RBCs (Dietrich et al., 2000). The released ATP then binds to P2Y receptors on the endothelium and stimulates vasodilatation. Thus, RBC function as an O_2 sensor, contributing to the regulation of blood flow and O_2 delivery, by releasing ATP depending on the oxygenation state of haemoglobin. ADP activates a negative feedback pathway for ATP release from human RBCs via $P2Y_{13}$ receptors (Wang et al., 2005). Since blood consists of approximately 40% RBCs that contain a 1,000-fold higher ATP concentration than plasma (mmol/l vs μmol/l), even a minor release of ATP from the high intracellular concentrations could have major circulatory effects. A negative feedback system may therefore be of great physiological importance to mitigate ATP release.

1.3.2 Atherosclerosis

Atherosclerosis is the main cause of ischaemic stroke and cardiovascular disease and is now considered to be an inflammatory disease (Hansson, 2005). Evidence both from basic research and from

clinical studies indicates important involvement for purinergic signalling in the atherosclerotic process at several levels.

1.3.2.1 Atherosclerosis-P2 receptor-mediated effects on inflammatory cells

Inflammatory cells express a large number of P2 receptors with multiple effects (Di Virgilio et al., 2001). The final result is difficult to predict depending on the subtype of receptor expressed by a particular cell type and on the differentiation stage of the cell. The most important inflammatory cells for atherosclerosis are monocytes that differentiate into macrophages or dendritic cells in the plaque, and the T-helper and suppressor lymphocytes that coordinate the inflammatory reaction in the plaque (Hansson, 2005). $P2X_7$, $P2Y_2$ and $P2Y_{11}$ receptors have been suggested to be important players in atherosclerosis (Di Virgilio and Solini, 2002). The $P2X_7$ receptor is mitogenic and anti-apoptotic for T lymphocytes (Kawamura et al., 2005), is important for release of interleukin (IL)-1 (Ferrari et al., 1997), tumour necrosis factor (Suzuki et al., 2004) and L-selectin, an adhesion molecule important for lymphocyte binding to endothelium (Gu et al., 1998). All of these effects are known to be important for atherosclerosis development. ATP and UTP are chemotactic for dendritic cells probably via the $P2Y_2$ receptor and may attract inflammatory cells to the vascular lesion (Idzko et al., 2002). The $P2Y_2$ receptor enhances the oxidative burst in human macrophages (Schmid-Antomarchi et al., 1997). A polymorphism (G-459-A) in the $P2Y_{11}$ receptor has been shown to have clinical importance by increasing the risk of acute myocardial infarction (AMI) (Amisten et al., 2007). The G-459-A, carried by one fifth of the population,

causes an Ala-87-Thr substitution in the $P2Y_{11}$ ATP receptor and increases the risk of myocardial infarction by 21%. The mechanism by which the polymorphism causes AMI seems to be coupled to increased inflammation because the Thr-87 variant of the $P2Y_{11}$ receptor was coupled to elevated C-reactive protein (CRP) levels. CRP is a marker of inflammation and an independent prognostic risk factor for the development of AMI (Hansson, 2005). ATP acting on $P2Y_{11}$ receptor regulates the maturation of human monocyte-derived dendritic cells and induces immunosuppression by inhibiting T-helper cytokines and promoting T-helper 2 cytokines (Kaufmann et al., 2005). $P2Y_{11}$ receptor is important in the development of atherosclerosis via modulation of inflammation either via effects on T lymphocytes or macrophages cells. UTP and ATP stimulate expression of proinflammatory vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells through activation of the $P2Y_2$ receptor and increase the adherence of monocytic cells to human coronary endothelial cells, an effect that is inhibited by anti-VCAM-1 antibodies (Seye et al., 2003). VCAM is important for the recruitment of monocytes and lymphocytes. In conclusion, ATP and UTP stimulate several inflammatory responses known to be important for atherosclerosis development.

1.3.3 Heart

Adenine nucleotides are continually present in quite variable amounts in the extracellular space of the heart. ATP is released in the heart as a cotransmitter together with catecholamines from sympathetic nerves, but it may also be released from other sources in the heart such as endothelium, platelets, RBCs and ischaemic myocardium

(Vassort, 2001). P2 receptors are abundantly expressed in the foetal human heart (Bogdanov et al., 1998) as well as in the adult human heart of both healthy subjects and patients with heart disease (Banfi et al., 2005; Vassort, 2001).

1.3.3.1 Inotropy

In cardiomyocytes, ATP stimulates a pronounced positive inotropic effect and may also act in synergy with β-adrenergic agonists to augment myocyte contractility (Danziger et al., 1988; Vassort, 2001; Zheng et al., 1996) ATP stimulates an increase in cytosolic calcium and evidence for the involvement of inositol 1,4,5-trisphosphate (IP3)-coupled $P2Y_2$ receptors and ion channel P2X receptors has been presented (Mei and Liang, 2001; Podrasky et al., 1997; Scamps and Vassort, 1994). The inotropic effects of ATP are dependent on both IP3 and cAMP (Balogh et al., 2005). Catecholamines act on β-receptors and mediate their inotropic effect by an increase of cAMP; antagonists of these receptors are important drugs for the treatment of hypertension and reduce mortality in congestive heart failure. UTP has been shown to induce a positive inotropic effect in rat atria and in rat and guinea pig ventricular cardiomyocytes (Froldi et al., 1994; Froldi et al., 2001). Stable UTP and UDP analogues induce a pronounced inotropic effect on mouse cardiomyocytes (Wihlborg et al., 2006). The $P2Y_2$ receptor is the most abundantly expressed receptor with very low levels of the $P2Y_4$ receptor in the human heart, suggesting that the inotropic effects of UTP are mediated via $P2Y_2$ receptors, while the UDP effects are mediated via the $P2Y_6$ receptor (Wihlborg et al., 2006). These mechanisms are mediated via IP3-mediated signalling. P2X receptors are probably also involved, since

several subtypes are expressed, but it has not been possible to perform a clear receptor characterisation due to lack of selective antagonists.

1.3.3.2 Myocardial infarction

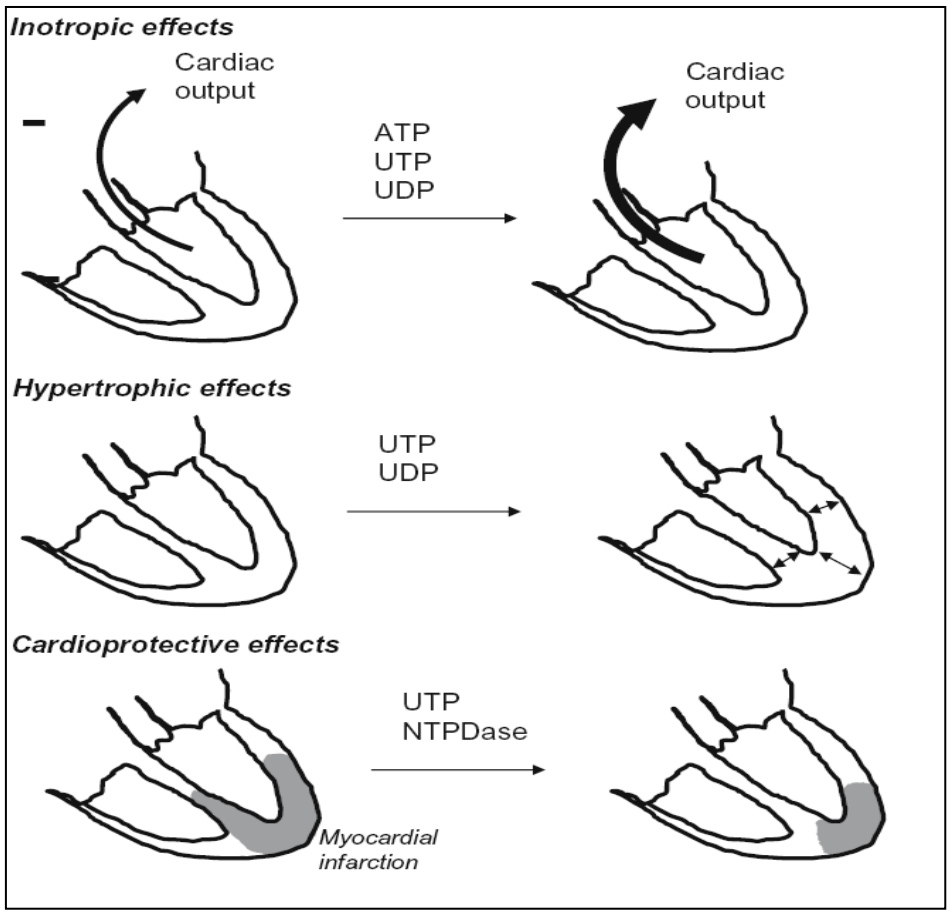
Using microdialysis, ATP in the interstitial space has been estimated to be around 40 nmol/l, but the levels may increase markedly during electrical stimulation, ischaemia, challenge with cardiotonic agents, increase in blood flow, mechanical stretch and increased work load (Vassort, 2001). ATP is released from cardiomyocytes during reduced oxygen tension and both UTP and ATP are released from the heart during cardiac ischaemia (Erlinge et al., 2005). It is noteworthy that patients with myocardial infarction have higher plasma levels of both ATP and UTP (Borna et al., 2005; Wihlborg et al., 2006). There is a significant increase in ectonucleotidase activity (NTPDase1) in the hearts of patients with ischaemic heart disease (Kittel et al., 2005) that could represent a compensatory mechanism against increased nucleotide levels during chronic ischaemia. An interesting yin and yang situation for ATP and UTP has been revealed regarding hypertrophic effects: UTP, but not ATP, causes hypertrophic growth in neonatal cardiomyocytes (Figure 1.5) (Zheng et al., 1994). In contrast, ATP inhibits hypertrophy and may even induce apoptosis and necrosis (Mazzola et al., 2008). Both UTP and ATP transactivate epidermal growth factor receptors, but only ATP stimulates the hypertrophic marker genes atrial natriuretic peptide and myosin light chain 2 (Morris et al., 2004). Similarly, UTP but not ATP protects cultured cardiomyocytes against hypoxic stress (Yitzhaki et al., 2005). Since UTP is released during preconditioning (Erlinge et al.,

2005), a role for this nucleotide in the protective effects of preconditioning is plausible. Recently, Yitzhaki and co-workers were able to demonstrate prominent reductions in myocardial infarction size and improved rat heart function *in vivo*, by a single intravenous bolus dose of UTP before ischaemia (Yitzhaki et al., 2005) (Figure 1.5).

1.3.3.3 Congestive heart failure

There are several reports of alterations or adaptations of purinergic signalling during congestive heart failure. The positive inotropic effect of ATP is impaired in heart failure, but reversed by the angiotensin-converting enzyme inhibitor imidapril (Saini et al., 2005). The contractile responses for the $P2Y_{11}$ receptor agonist AR-C67085 are decreased in heart failure, suggesting a downregulation of this receptor function in cardiomyocytes in a similar manner as seen for β1-receptors in congestive heart failure (Balogh et al., 2005). In human hearts, only the $P2X_6$ receptor was altered in congestive heart failure (Banfi et al., 2005). In congestive heart failure, $P2X_1$ receptor is downregulated in VSMC in resistance arteries, which could represent a protective response against the increased sympathetic nerve activity and peripheral resistance seen in congestive heart failure (Malmsjo et al., 1999). Several P2 receptors have been suggested as targets for pharmacological treatment of congestive heart failure. Overexpression of $P2X_4$ receptors has a beneficial, lifeprolonging effect in a heart failure model (Yang et al., 2004). The extracellular pyrimidines UTP and UDP may be inotropic factors in man, acting on $P2Y_2$ and $P2Y_6$ receptors stimulating the same intracellular pathways as angiotensin II. Synthetic agonists could thus

be used as inotropic agents during circulatory shock and antagonists may have effects similar to angiotensin II receptor blockers, being beneficial in the treatment of hypertension and congestive heart failure (Erlinge and Burnstock, 2008).



F***igure 1.5: Functional role of purines and pyrimidines acting on P2 receptor in the regulation of the heart***

ATP, UTP and UDP exert inotropic effects on cardiomyocytes leading to increased cardiac output. UTP and UDP stimulate hypertrophy of cardiomyocytes, while ATP can have apoptotic effects. UTP protects against ischaemic injury and cardiomyocyte cell death (modified from Erlinge and Burnstock, 2008).

1.3.4 Platelets

ADP, released from erythrocytes or produced after ectonucleotidase breakdown of ATP released from erythrocytes and endothelial cells, was found to cause platelet aggregation as early as in 1961, before the concept of P2 receptors was conceived (Gaarder et al., 1961). Two ADP receptors ($P2Y_{12}$ and $P2Y_1$) and one ion channel ATP receptor ($P2X_1$) are expressed on platelets (Kunapuli et al., 2003). The $P2Y_{12}$ receptor is coupled to inhibition of cAMP. The molecular identifications of the $P2Y_{12}$ receptor and generation of knockout mice revealed highly prolonged bleeding times in the absence of $P2Y_{12}$; the platelets of these mice aggregated poorly in response to ADP and displayed a reduced sensitivity to thrombin and collagen (Foster et al., 2001). The $P2Y_1$ receptor is responsible for ADP-induced shape change and weak, transient aggregation, while the $P2Y_{12}$ receptor is responsible for the completion and amplification of the response to ADP and to all platelet agonists, including thromboxane A2, thrombin and collagen. A synergistic interaction between ATP and NA in stimulating platelet aggregation may have significant clinical implications and suggests a prothrombotic role for ATP in stress (Birk et al., 2003). ADP acting on $P2Y_{12}$ receptors is not only important for platelet activation, but also stimulates vasoconstriction. Stable drugs with antagonistic effects on $P2Y_{12}$ receptors, affecting both platelets and VSMC, could be of double therapeutic benefit in their prevention of both thrombosis and vasospasm (Wihlborg et al., 2004).

1.3.4.1 Coagulation

The prothrombotic effect of ADP on the platelets is counteracted by the effects of UTP and ATP on the endothelium that stimulate substained release of tissue-type plasminogen activator (tPA) with fibrinolytic effects (Hrafnkelsdottir et al., 2001). ATP also releases plasminogen activator inhibitor (PAI-1) which, in turn, inhibits tPA (Bouchie et al., 2000). Thus, P2 receptors are involved in several stages of haemostasis, which is important for the development of atherosclerosis and thrombotic occlusions leading to myocardial infarction and stroke. The first ADP inhibitors, ticlopidine and clopidogrel, were developed before the cloning and identification of the platelet ADP receptors. Later, it was concluded that they were prodrugs, converted in the liver to a metabolite that binds irreversibly to the $P2Y_{12}$ receptor resulting in non-competitive antagonism. Ticlodipine has the disadvantage of a small risk of neutropaenia, so clinically so far the most important contribution of drug development aimed at P2 receptors has been the beneficial effects of the platelet ADP receptor antagonist Clopidogrel in atherosclerotic disease. In the CAPRIE trial, Clopidogrel was even more effective compared to aspirin in preventing myocardial infarctions (Logman et al., 2010). Clopidogrel, in addition to aspirin, turned out to be necessary to prevent acute in-stent thrombosis in percutaneous coronary interventions. The CURE trial demonstrated that the addition of clopidogrel to aspirin reduced clinical events by 20% in acute coronary syndromes (Mehta et al., 2001). This has also been shown for ST-elevation myocardial infarctions (COMMIT and CLARITY trials) (Berg et al., 2007). However, long-term secondary prevention in high-risk groups with clopidogrel added to aspirin did not have significant benefits (CHARISMA trial) (Berger et al., 2010).

Clopidogrel is a rather weak antagonist at $P2Y_{12}$ receptors with variable effects, often referred to as clopidogrel resistance. Newer, $P2Y_{12}$ receptor antagonists, such as prasugrel and AZD6140 (Ticangrelor), have a stronger and more consistent effect and are currently being studied in major clinical trials (TRITON and PLATO)(Serebruany et al.,2010). Clopidogrel is one of the most prescribed drugs in the world for the treatment of thrombosis, stroke and myocardial infarctions in millions of patients. Due to its patent expiration in 2011, it is believed that the new drugs under development will take part of its market starting from that date.

2
AIM OF STUDY

It is now widely accepted that, in addion to functioning as intracellular energy source, extracellular nucleotides initiate a wide

range of intracellular signalling cascades through the activation of a plethora of purinergic receptors. The purines and pyrimidines ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP), uridine diphosphate (UDP), and, as more recently recognized, sugar nucleotides like UDP-glucose and UDP-galactose (Abbracchio et al., 2006; Burnstock, 1997) act on various P2X homo- and heteromultimer ionotropic receptors and on 8 P2Y metabotropic receptor subtypes (Ralevic and Burnstock, 1998). In the cardiovascular system, evidence is accumulating to indicate that P2 receptors mediate important physiologic effects, including vasoconstriction and vasodilatation, growth of vascular smooth muscle and endothelial cells, angiogenesis, vascular remodelling, coagulation, platelet aggregation, inflammation and several aspects of cardiac function. The purinergic system and its receptors are also involved in development of myocardial infarction, heart failure and xenograft rejection (Erlinge and Burnstock, 2008; Vassort, 2001). The normal basal levels of plasma and interstitial ATP are very low and do not exceed 20-40 nM in human venous plasma and in the cardiac interstitial space. Hypoxia and ischemia induce ATP release into the cardiac interstitial space from purinergic nerves innervating the heart, cardiac vascular endothelial cells and cardiomyocytes. ATP is also released from the cytoplasmatic pool under these conditions. In cardiomyocytes cells, ATP acting through P2Y and P2X receptors stimulates a pronounced positive inotropic effect and may also act in synergy with ß-adrenergic agonists to augment myocyte contractility. In addition, potent effects may be exerted by nucleotides on cardiomyocytes viability, under both physiological conditions and after hypoxia, when cardiomyocytes themselves release massive

amount of ATP, mainly by extrusion via the maxi-anion channel (Dutta et al., 2008). In the last years, we have investigated the effects of both adenine and uracil nucleotides on the viability of HL-1 cardiomyocytes, the only available cell line that spontaneously contracts in vitro and maintains a differentiated cardiac phenotype. We showed that murine HL-1 cardiomyocytes express a wide panel of P2X and P2Y receptors known that either exclusively respond to adenine nucleotides (P2X receptors), to both adenine and uracil nucleotides ($P2Y_2$, $P2Y_4$, $P2Y_6$) or to sugar nucleotides ($P2Y_{14}$ receptor). We demonstrated that the exposure of cardiomyocytes to high concentrations of adenine nucleotides (ATP, ADP or BzATP) induces cardiomyocyte cell death through a mechanism involving both P2Y and P2X receptors (Mazzola et al., 2008). However, in that study the specific P2 receptors involved were not identified. It would be very interesting to study the role of specific P2 receptor in the cell death associated to cardiac ischemia. On this basis, a first aim of the present study has been to set up and characterize a suitable ischemic/hypoxic protocol of cardiomyocyte ischemia in HL-1 cells. A second goal of this study has been to assess if ATP is released from these cells after an ischemic/hypoxic stress. A third goal has been to establish whether specific P2 receptors play a role in the cell death associated to cardiomyocyte ischemic/hypoxic stress. To do so we have taken advantage of both pharmacological agents and of silencing techniques via small interfering RNAs.

3 MATERIALS AND METHODS

3.1 Cell culture

HL-1 cells, a cardiac muscle cell line derived from the AT-1 mouse atrial myocyte tumor lineage, were a kind gift from William C. Claycomb, Ph.D. (Professor of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, Louisiana). HL-1 cells are currently the only available cardiomyocyte cell line that continuously divides and spontaneously contracts while maintaining a differentiated cardiac phenotype. Cell were cultured in complete Claycomb medium supplemented with 10% foetal calf serum (FCS) and Norepinephrine (100 μM), following Professor Claycomb's instructions. (White et al., 2004). They were used for experiments when approximately 70 to 80% confluent.

3.1.1 Treatment of cell

In separate experimental groups, cells received no intervention (normoxia control, 95% air and 5% CO_2) or were exposed to ischemic/hypoxic stress. Hypoxia was produced by exposure to 5% CO_2 and 95% N_2 in a modular incubator chamber (Billups-Rothenberg) for 16 h in the presence of serum- and glucose-free Dulbecco's Modified Eagle Medium (DMEM). Pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS, 100 μmol/L; suramin (100 μmol/L); gadolinium (III) chloride ($GdCl_3$ 100 μmol/L); 2′,3′-O-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate monolithium trisodium salt (TNP-ATP, 10 μmol/L); 4-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1 piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester (KN-62, 10 μmol/L); (5-[[5-3,4-dihydro-2-oxo-4-thioxo-1(2H)-pyrimidinyl]methyl]-N-[1Htetrazol-5-yl]-2-furancarboxamide

(AR-C118925, kind gift from Prof. C.E. Muller); N,N''-1,4-butanediylbis[N'-(3-isothiocyanatophenyl) thiourea (MRS2578 1-10 μmol/L); apyrase (30 U/mL); pertussis toxin (PTX, 100 nmol/L); GF 109203X (10 μmol/L, Sigma-Aldrich); guanosine 5′-[β-thio]diphosphate trilithium salt (GDP β-s 5 μmol/L), were all added to cultures 1 h before hypoxia.

3.2 Real-time reverse transcription polymerase chain reaction

Total cell RNA was extracted using TRIzol Reagent and reverse transcribed at 42°C for 50 min and 70°C for 15 min (Perkin-Elmer Life Sciences, Milano, Italy). For 1 μg of total cell RNA, we used 200 units of reverse transcriptase (RT; SuperScript II, Invitrogen, Life Technologies, Milano, Italy), 3 μg of random hexamers primers, 1 mmol/L of dNTPs and 40 units of Rnase inhibitor. Real-time quantitative polymerase chain reaction (PCR) was then carried out to detect $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2X_7$ mRNA. 18S rRNA was used for sample .

The sequences of the primers used were:

- $P2Y_6$ sense: 5´- CCC AAC CTG CCT TGA AAA CA-3´,
- $P2Y_6$ antisense: 5´- TCG GAG AGT CTG TCT CAT GCA A-3´;
- 18S sense: 5'-CGGCTACCACATCCAAGGAA-3';
- 18S antisense: 5'-CCTGTATTGTTATTTTTCGTCACTACCT-3'.

Primers for the detection of $P2Y_2$ (QT00097202), $P2Y_4$ (QT00266686), and $P2X_7$ (QT00130900) were from Qiagen (Milan, Italy). A total of 2.5 μL of cDNAs were incubated in 25 μL IQ Supermix containing $P2Y_2$, $P2Y_6$, $P2X_7$, or 18S primers and

SYBRGreen fluorescence dye (Bio-Rad Laboratories, Milano, Italy). Real-time reverse transcription PCR was carried out in triplicate for each sample on an iCycler Optical System, Bio-Rad Laboratories (Milano, Italy). Amplifications of $P2Y_4$ were performed in a GeneAmp 9700 thermal cycler (Applied Biosystems) for 30-40 cycles (94°C for 45 s, 45 s at an annealing temperature of 51-60°C, 72°C for 45 s), after denaturation at 94°C for 2 min. Amplified product was size-separated by electrophoresis on a 1.5% agarose gel.

3.3 RNA interference and cell transfection

Validated high-performance purity grade small interfering RNAs (siRNA) against mouse $P2Y_2$ (SI00179235), $P2Y_4$ (SI00217588), $P2Y_6$ (SI01367891), and $P2X_7$ (SI00197330) were synthesized by QIAGEN (Milan, Italy) using the HiPerformance siRNA design algorithm and a proprietary homology analysis tool. Control siRNA, with a nonsilencing oligonucleotide sequence that does not recognize any known homology to mammalian genes, was also generated as a negative control. RNA, dissolved in an appropriate volume of RNAase-free water. HL-1 cardiomyocytes, at 70-80% confluence, were transfected with siRNA using HiPerFect Transfection Reagent (Qiagen, Milan, Italy). After 24 h, the transfection procedure was repeated before induction of ischemic/hypoxic stress .

3.4 Nuclear staining of adhering cells

After cell treatment, cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and nuclear chromatin stained by using the fluorescent dye Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA).

3.5 Flow cytometric evaluation of apoptosis

The percentage of necrotic cells in the adhering cells was evaluated by means of propidium iodide (PI) staining of DNA followed by flow cytometric analysis. When employed at isotonic conditions, PI can indeed only permeate cells with damaged membranes, thus providing a direct measure of earlier membrane events associated to necrosis. Induction of apoptosis was also confirmed by measuring the switch out of Annexin V binding to phosphatidylserine on outer membrane of apoptotic cells .

3.6 Nucleosome immunoassay

Photometric enzyme immunoassay for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death was performed. (Roche Diagnostics GmbH Roche Applied Science 68298 Mannheim Germany).

3.7 ATP release assay

ATP release was assayed by means of the ATP Bioluminescence Assay Kit HS II (Roche Diagnostics GmbH Roche Applied Science 68298 Mannheim Germany), an ATP monitoring system based on firefly (*Photinus pyralis*) luciferase. The ATP assay system is based on the production of light caused by the reaction

of ATP with added luciferase and D-luciferin. This is illustrated in the following reaction scheme:

ATP + D-Luciferin + O_2 $\Rightarrow$Oxyluciferin +Mg^{2++} AMP + PPi + CO_2 + Light.

The emitted light is proportional to the ATP concentration was monitored at 560 nm using a luminometer.

3.8 Statistical analysis

All results are expressed as mean ± S.E.M. of at least three independent experiments. Statistical significance between groups was derived using a paired 2-tailed Student's t test. Differences were considered significant when p was less than 0.05.

4
RESULTS

4.1 HL-1 cardiomyocytes express P2 receptors responding to both adenine and uracil nucleotides

As a first step to the characterization of the effects of P2 receptors on the viability of HL-1 cells, we assessed the presence of all rodent P2 receptors by RT-PCR. Amplification products with the expected molecular weight for all known P2X receptors (with the only exception of $P2X_2$) and for $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ were obtained (Figure 4.1). No specific signals for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ were obtained; negative data were not due to technical problems, since specific amplification products for these receptors were detected in murine tissues or cell lines which express these receptors here utilized as positive controls (Figure 4.1).

4.2 Ischemic/Hypoxic stress induces ATP release in HL-1 cardiomyocytes

Cardiomyocytes release ATP in the extracellular milieu during cardiac ischaemia. Released cellular ATP can serve as a paracrine signaling molecule for intercellular communication, or can act in an autocrine manner to regulate cellular functions, including those of cardiomyocytes. Cultured HL-1 cardiomyocytes exhibited a basal release of ATP that was significantly increased at 30 minutes after the induction of an ischemic/hypoxic stress (mean increase of 63.6% ± 9.7; $p< 0.005$, n=3 from 3 independent experiments, Figure 4.2A). To ensure that, at this time point, membrane damage did not contribute to ATP release, we analyzed the conditioned media from ischemic/hypoxic cells for the presence of the cytosolic enzyme lactate dehydrogenase (LDH), a marker of cellular damage. We consistently found that ischemic/hypoxic-

induced ATP release occurred in the absence of LDH release, hence with no significant membrane damage compared with normoxic controls, suggesting that, at this early stage of hypoxia, ATP does not derive from cell necrosis but from release via transmembrane channels/pumps.

4.3 Ischemic/hypoxic stress promotes apoptosis of HL-1 cardiomyocytes

Ischemic/hypoxic stress induced significant increases in the appearance of HL-1 cardiomyocytes apoptosis, as assessed by two complementary assays including annexin V and nucleosomal immunoassay. An increased number of annexin-positive cardiomyocytes was registered after 16 hours of exposure to ischemic/hypoxic stress (Figure 4.2B). We found that, at this time after ischemic/hypoxic stress induction, the number of propidium iodide-positive cells was also increased compared to control value (+72.2 % ± 9.9, $p < 0.005$). To confirm the membrane-related changes associated with apoptosis, we performed nucleosomal ELISA assay, which detects later stages of apoptosis (i.e., internucleosomal DNA cleavage). ELISA analysis showed a strong increase in the apoptotic index, up to 320.9 % ± 15.8 of control value at 16 h (Figure 4.2C).

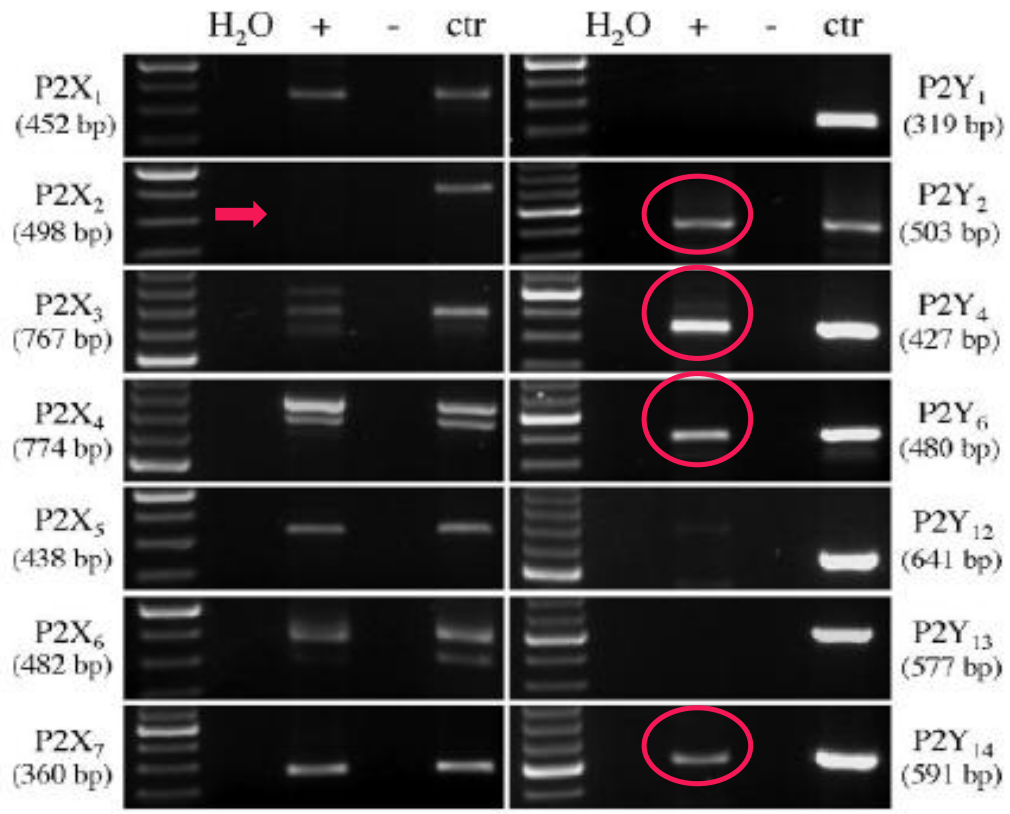


Figure 4.1: *HL-1 cardiomyocytes express $P2X_{1,3,4,5,6,7}$ and $P2Y_{2,4,6,14}$. The presence of P2 receptors was assessed using ad hoc designed RT-PCR primers for all cloned murine P2 receptors. For each receptor, amplification products in parallel with MW standards are reported. Various tissues or cell lines were analysed in parallel as a positive controls: trigeminal ganglia for $P2X_2$ and $P2X_3$, total brain for $P2X_1$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_{13}$ and $P2Y_{14}$, heart for $P2X_6$, liver for $P2Y_4$, lung for $P2Y_2$ and N9 cells for $P2Y_1$, $P2Y_6$ and $P2Y_{12}$.*

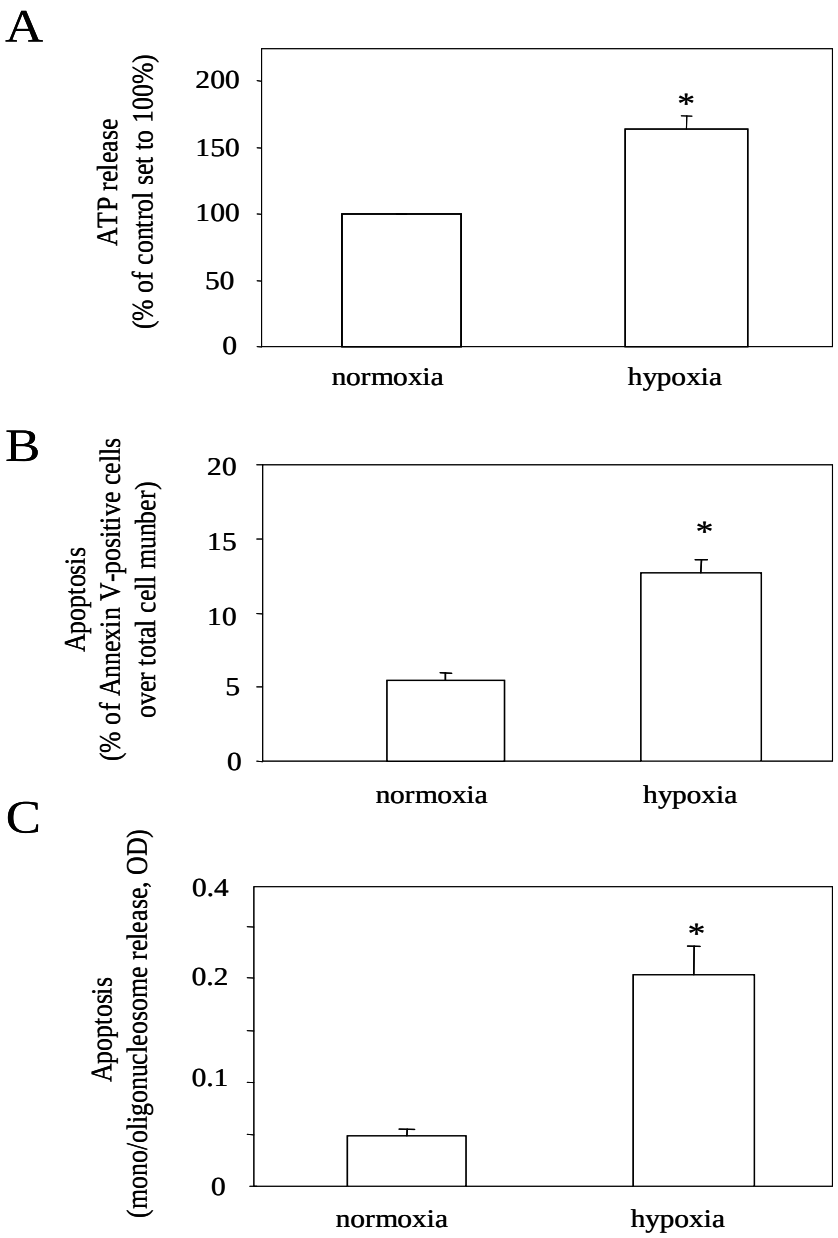


Figure 4.2*: *Ischemia/hypoxia induces release of ATP and promotes cardiomyocytes apoptosis: *ATP is released by HL-1 cells very early during hypoxia, which induces apoptosis.* ***A****, HL-1 cells were exposed to normoxia or hypoxia for 30 minutes; at the end of the incubation, ATP was measured in the conditioned medium. Results are the mean ± S.E.M., n=4, *p<0.05, Student "t" test.* ***B****,* ***C****, HL-1 cells were exposed to normoxia or hypoxia for 16 hours. At the end of the incubation, apoptotic cells were quantified by flow cytometric analysis as annexin V positive cells (****B****) or by immunoenzymatic determination of mono/oligonucleosome released in the cultured medium (****C****). Results are the mean ± S.E.M. from six separate experiments performed in triplicate. *p< 0.05, $ p<0.01 versus normoxic controls, Student "t" test.*

4.3.1 *Morphological features of apoptosis*

Because one important sign of apoptosis is the appearance of specific morphological changes, including nuclear condensation and chromatin fragmentation, we used Hoechst 33258, a fluorescent DNA-binding dye, to stain nuclear chromatin in HL-1 cardiomyocytes, and examined cell morphology by phase-contrast microscopy. Figure 4.3A and 4.3B show the phase contrast morphology of HL-1 cardiomyocytes exposed to normoxia or to ischemic/hypoxic stress. Control cultures have normal cell bodies and intact features with nuclei homogeneous in size and shape and very lightly stained with Hoechst 33258 (Figure 4.3A). However, when HL-1 cardiomyocytes were exposed to ischemia/hypoxia in a glucose-free medium for 16 h, we noticed morphologic alterations typical of cells undergoing apoptosis, which became rounded and condensed and lost adhesion from the monolayer. Furthermore, HL-1 cells displayed condensed chromatin that was brightly and uniformly stained by Hoechst 33258 and ranged in shape from a single uniform sphere to a collection of multiple chromatin dots typical of fragmented apoptotic nuclei (Figure 4.3B).

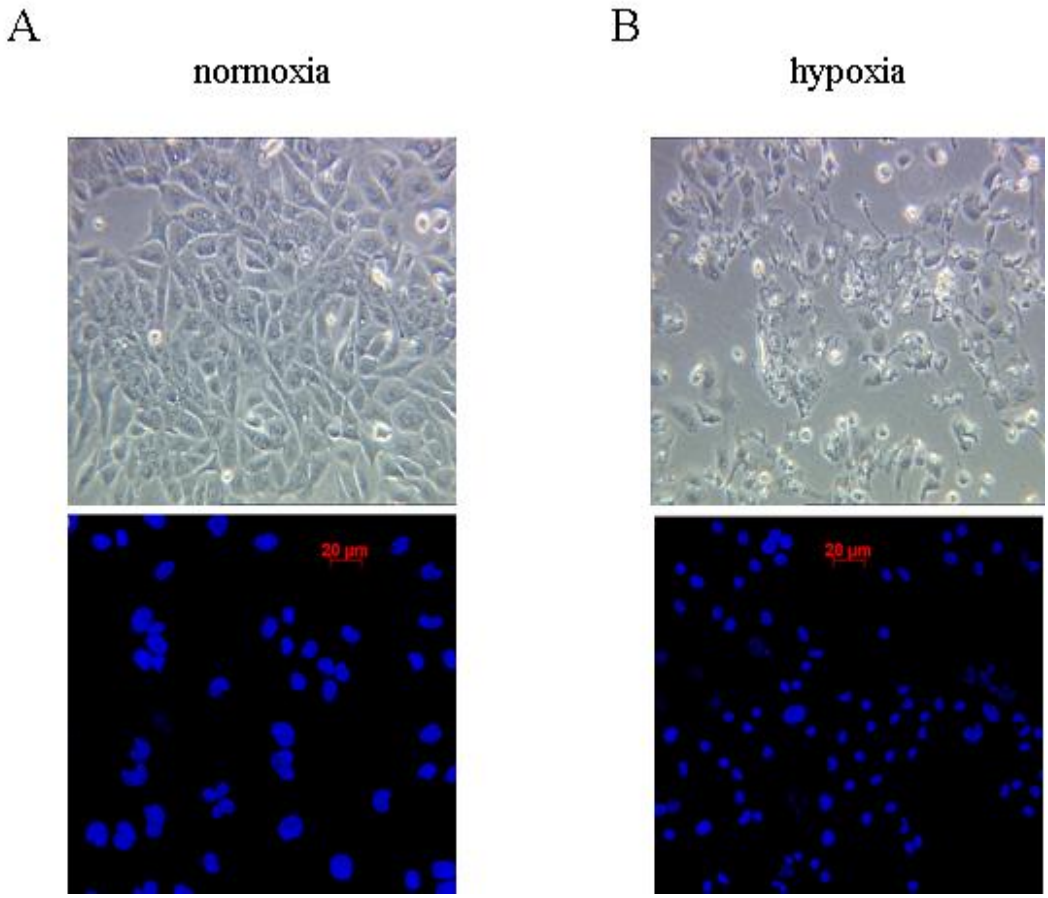


Figure 4.3: Cardiomyocytes show typical features of apoptosis: *A and B show representative images of HL-1 cells exposed to either normoxia or hypoxia for 16 h. Upper immages are phase-contrast micrographs; lower images refer to cultures stained with Hoechst 33,258. Under control conditions (A), HL-1 cells morphology appeared normal and cell density was approaching confluence. After exposure to ischemia/hypoxia in a glucose-free medium (B), many of the cells became rounded, showed condensed nuclei and detached from the culture dish.*

4.4 Effect of apyrase on ischemia/hypoxia-induced release of ATP and on cardiomyocyte apoptosis

Endogenously released ATP plays an important role in mediating HL-1 cardiomyocytes apoptosis, as treatment with apyrase, a nucleotidase that degrades nucleotide triphosphates into nucleotide monophosphates and thus completely abolishes the amount of ATP released in the medium during ischemia/hypoxia stress (Figure 4.4A), significantly prevented the induction of apoptosis (Figure 4.4B). Since there is, as expected, a basal release of ATP even in the absence of hypoxia, apyrase also significantly reduced basal ATP levels during normoxia (Figure 4.4A), but this was not associated to any change in cell viability, to confirm that, under control conditions, ATP does not mediate cell death.

4.5 Effect of the maxi-anion channel inhibitor $GdCl_3$ on ischemia/hypoxia-induced cardiomyocyte apoptosis

In rat cardiomyocytes, ATP release is mainly mediated by the maxi-anion channel under ischemic or hypotonic conditions (Dutta et al., 2004). Based on these findings, we reasoned that ischemic/hypoxic stress may lead to ATP release from HL-1 cardiomyocytes through similar mechanisms. To examine this possibility, HL-1 cells were treated with the maxi-anion channel blocker $GdCl_3$. Treatment of cells with $GdCl_3$ completely prevented the induction of apoptosis induced by ischemic/hypoxia stress (Figure 4.5).

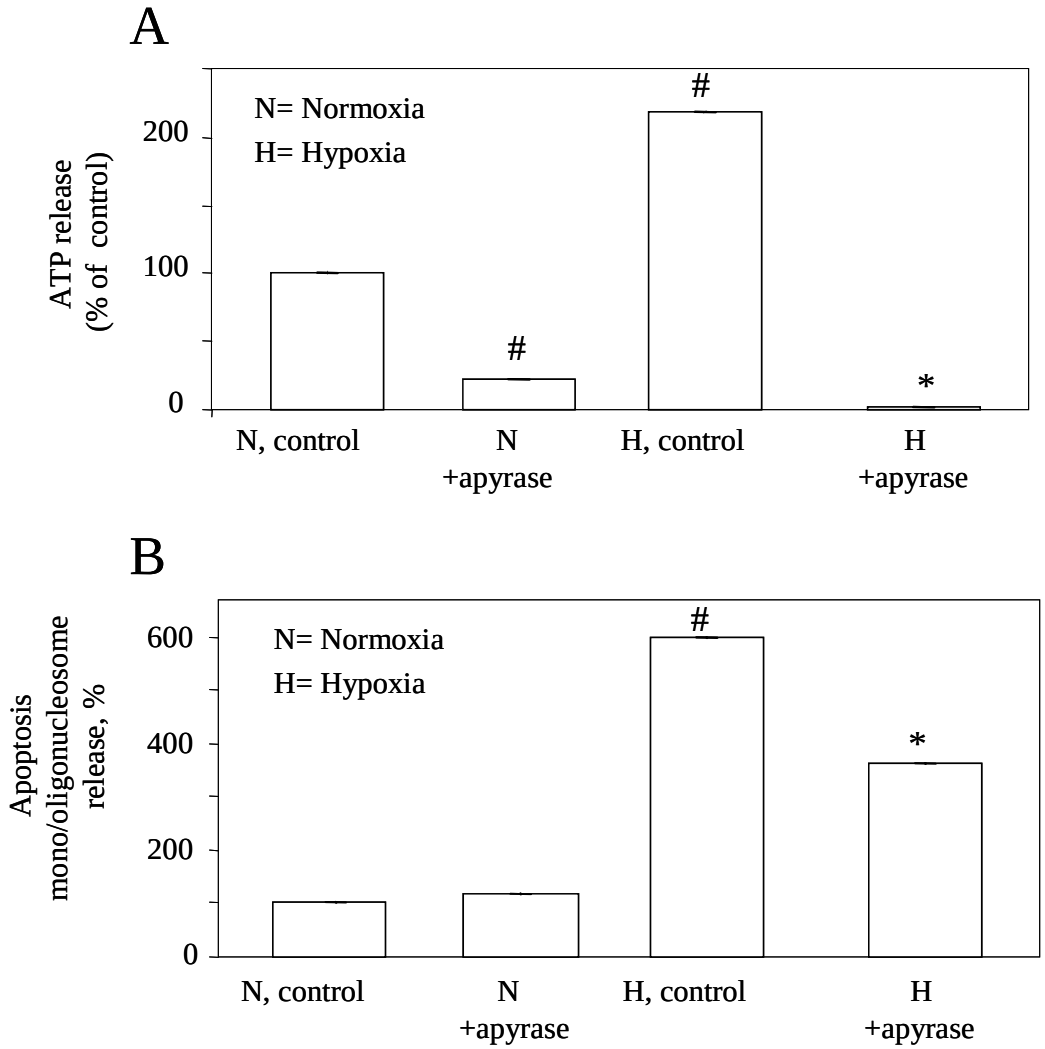


Figure 4.4: Apyrase inhibits ATP release and protects cardiomyocytes against cell death during ischemic/hypoxia period A, *Apyrase [30U/ml] was added to HL-1 cells immediately before normoxia (N) or hypoxia (H). After 30 min, ATP was measured in the medium. Results are the mean ± S.E.M., n=3, *p<0.05, Student "t" test.* ***B,*** *Apyrase [30U/ml] was added to cells immediately before a 16 h hypoxia (H). Parallel samples received apyrase under the same conditions and were maintained for 16 h under normoxic conditions (N). Control cells received vehicle. At the end of the incubation, enrichment of mono/oligonucleosome in cells was determined by ELISA assay. Values are the mean ± S.E.M. from 3 independent experiments run in triplicate; *p<0.05 versus corresponding untreated values, Student "t" test.#: p< 0.05 versus normoxia untreated cell Student "t" test. N, normoxia; H, hypoxia; H-N, values from hypoxia minus values from normoxia.*

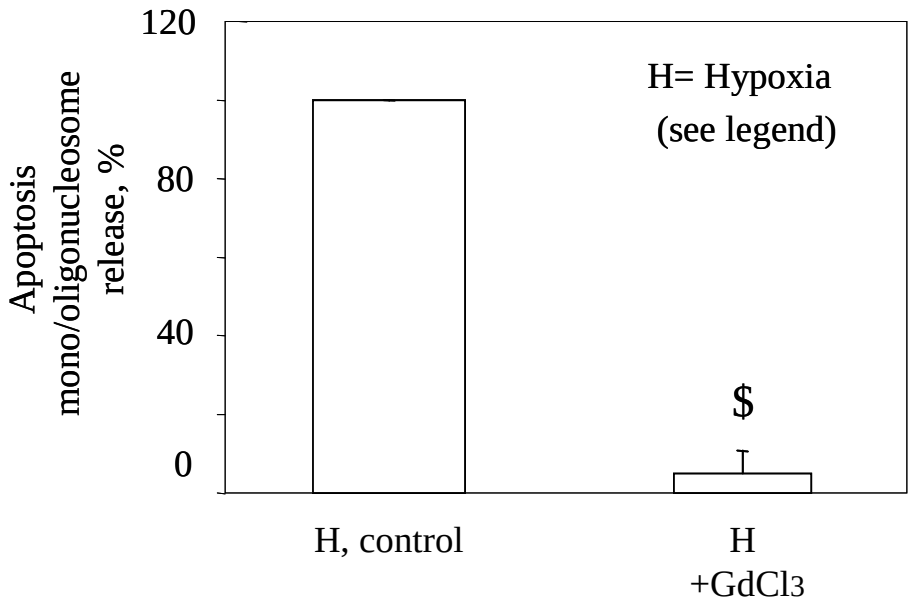


Figure 4.5: GdCl3 protects cardiomyocytes against cell death during ischemic/hypoxia period

GdCl3 (100µmol/L) was added to HL-1 cells 1 hr before normoxia or hypoxia. Hystograms report apoptosis under hypoxic conditions after subtracting the apoptosis value detected under normoxic conditions. At the end of the incubation, enrichment of mono/oligonucleosome in cells was determined by ELISA assay. Values are the mean ± S.E.M. from 3 independent experiments run in triplicate; $: p<0.01 versus corresponding control value (vehicle), Student "t" test

4.6 ATP treatment induces HL-1 cardiomyocyte apoptosis

To further confirm the role of released ATP in the induction of apoptosis by ischemia/hypoxia in HL-1 cells, we treated cardiomyocytes with different concentrations of ATP. We found that stimulation of HL-1 cardiomyocytes by ATP itself for 24 h at 250-500 μmol/L increased apoptosis up to 254% ± 37 of control values set to 100% (Figure 4.6).

4.7 P2 receptors antagonists prevent the appearance of HL-1 cardiomyocyte apoptosis induced by ischemia/hypoxia

We have previously shown that HL-1 cardiomyocytes express all known P2X receptors (except for $P2X_2$), as well as the $P2Y_{2,4,6,14}$ subtypes (Figure 4.1). To verify if the apoptotic effect of the ischemic/hypoxic stress was actually due to activation of P2 receptors by ATP or other nucleotides released during hypoxia, HL-1 cardiomyocytes were exposed to ischemic/hypoxic stress in the presence of non selective P2 receptor antagonists, such as PPADS and suramin. These treatments almost completely prevented the appearance of apoptosis (-82.7% ± 7.7 after PPADS; -68% ± 12 after suramin) (Figure 4.7). Because PPADS antagonises $P2X_{1,2,3,5}$ and $P2Y_{4,6}$, whereas suramin effectively blocks $P2X_{1,2,3,5}$ and $P2Y_{2,6,11}$, these data suggest that both P2X and P2Y receptors contribute to the cardiomyocytic apoptosis induced by ischemia/hypoxia. However, since P2X receptors can form heteromers characterized by different and unpredictable pharmacological profiles, a definite conclusion regarding the specific receptor subtypes involved cannot be drawn from these data.

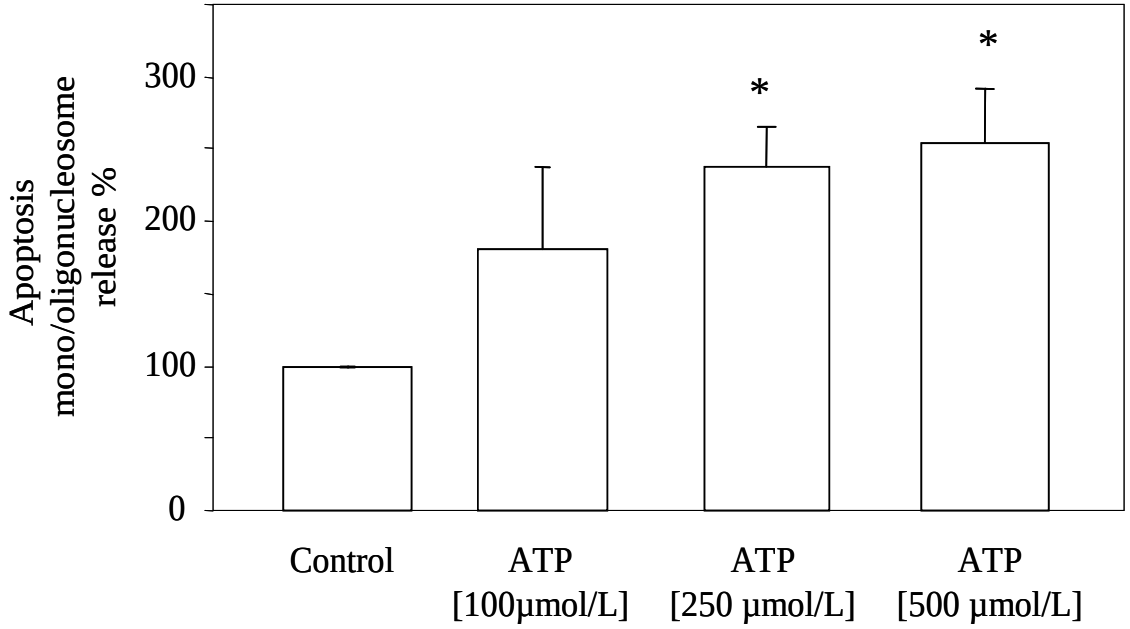


Figure 4.6: ATP itself induces apoptosis. *HL-1 cells were treated with either vehicle (Control) or ATP at the indicated concentrations for 24 h. At the end of the incubation, enrichment of mono/oligonucleosome in cells was determined by ELISA assay. Results are the mean ± S.E.M. from 3 independent experiments run in triplicate; *p<0.05 versus control untreated value, Student "t" test.*

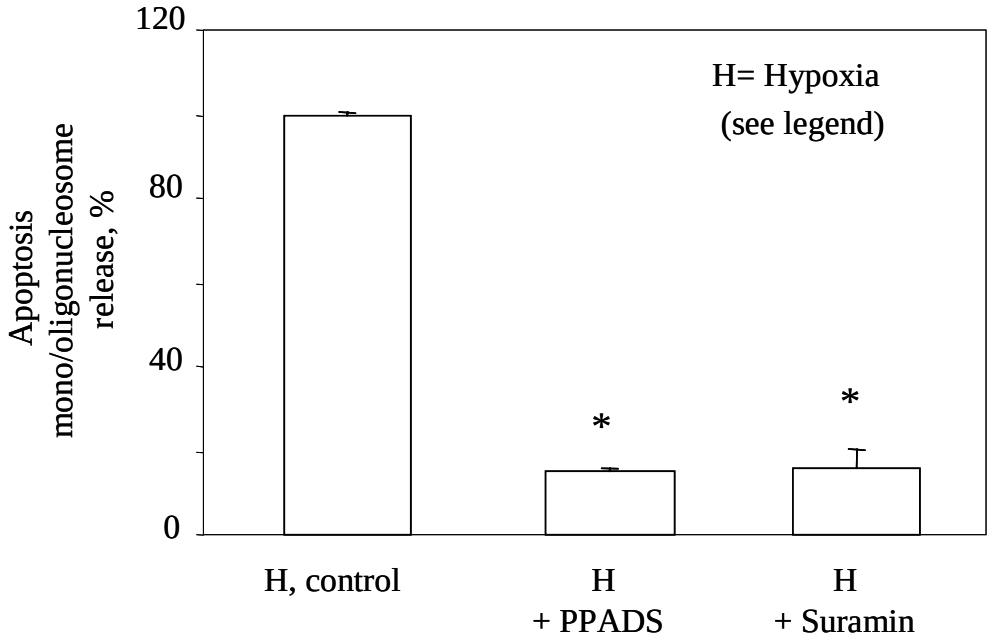


Figure 4.7: The non selective P2X/P2Y receptor antagonists PPADS and Suramin protect HL-1 cardiomyocytes from induction of apoptosis after ischemic hypoxia. *PPADS (pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate, 100µmol/L) and suramin (100µmol/L) were added to cells 1 h before normoxia or hypoxia. After 16 h, enrichment of mono/oligonucleosome in cells was determinated by ELISA assay. Values represent changes with respect to apoptosis found in control (vehicle) hypoxic cells set to 100% and are the mean ± S.E.M. of 3 independent experiments run in triplicate. *:p< 0.05 versus corresponding control, Student "t" test. H= apoptosis value under hypoxic conditions after subtraction of the apoptosis value detected under normoxic conditions.*

4.8 Inhibition of $P2X_7$ partially prevents the ischemia/hypoxia-induced apoptosis

Pre-treatment of cells with 2′,3′-O-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate monolithium trisodium salt (TNP-ATP), an antagonist for $P2X_{1–4}$ receptors, failed to significantly prevent the appearance of apoptosis induced by ischemic/hypoxic stress (Figure 4.8), thus ruling out a role for these P2X receptor subtypes in our model. By contrast, when the specific $P2X_7$ antagonist KN-62 was tested, a significant reduction of apoptosis in the ischemic/hypoxic condition was monitored (Figure 4.8). In order to validate the effect of the $P2X_7$ antagonist, we silenced the expression of $P2X_7$ receptor using small interfering (si) RNAs specifically designed to knock down this receptor subtype. Treatment of HL-1 cells with a $P2X_7$ receptor siRNA construct reduced the $P2X_7$ receptor mRNA levels by 54% ±3.4 (Figure 4.9A). The decrease in $P2X_7$ receptor expression resulted in a partial, albeit statistically significant, inhibition of apoptosis in response to the ischemic/hypoxic stress (-34%± 2.7) (Figure 4.9B). Taken together, these results point to a possible contribution of the $P2X_7$ receptor in the apoptosis in HL-1 cardiomyocytes induced by ischemia/hypoxia.

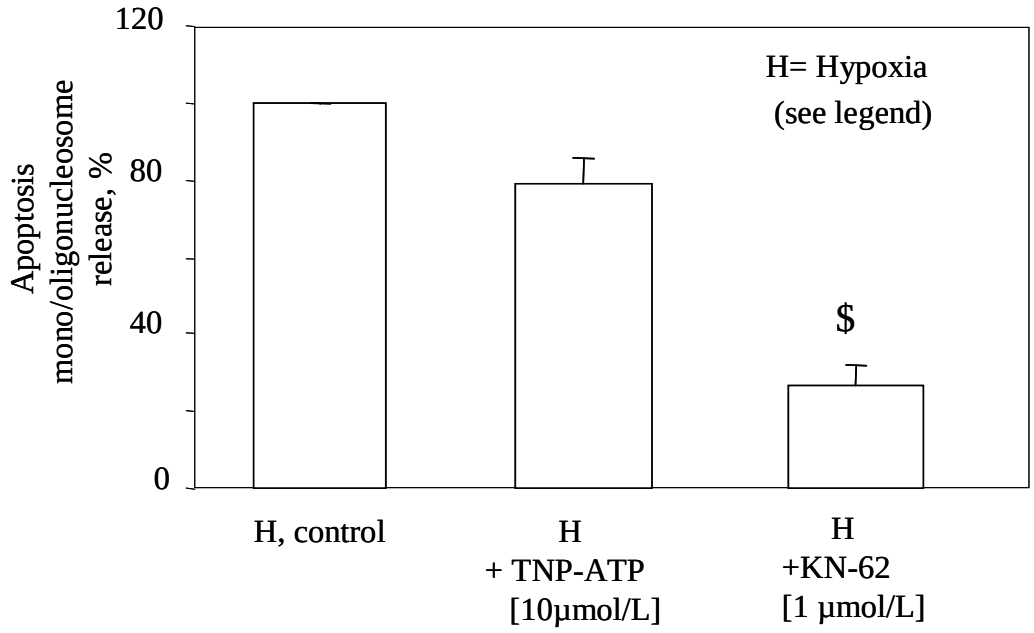


***Figure 4.8**: **Inhibition of $P2X_7$, but not of $P2X_{1-4}$ receptors, prevents HL-1 cardiomyocyte apoptosis induced by hypoxia** TNP-ATP (10µmol/L) or KN-62 (1µmol/L) were added to cells 1 h before normoxia or hypoxia. After 16 h, enrichment of mono/oligonucleosome in cells was determined by ELISA. Values represent changes with respect to apoptosis found in control (vehicle) hypoxic cells set to 100% and are the mean ± S.E.M. from 4 independent experiments run in triplicate; $: $p<0.01$ versus corresponding control values, Student "t" test. H= apoptosis value under hypoxic conditions after subtraction of the apoptosis value detected under normoxic conditions.*

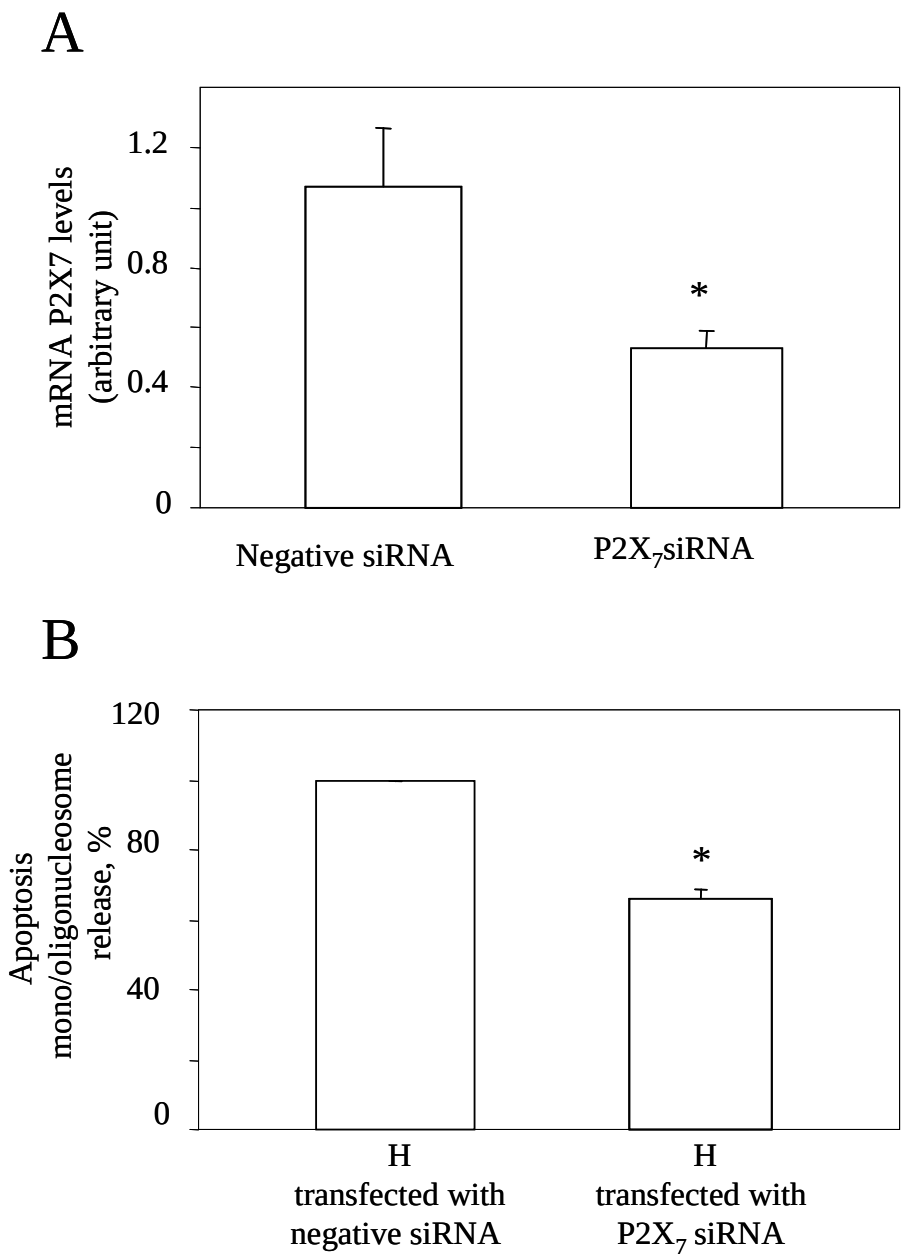


***Figure 4.9**: **Gene silencing of $P2X_7$ prevents HL-1 cardiomyocyte apoptosis induced by hypoxia A,** mRNA analysis of $P2X_7$ by qRT-PCR in HL-1 cells transfected with ineffective (negative) siRNA or with $P2X_7$ siRNA. **B,** HL-1 cells transfected with ineffective (negative) siRNA or with $P2X_7$ siRNA were exposed to normoxia or hypoxia for 16 h for detection of apoptosis. Apoptosis values were calculated in hypoxic cells and set to 100%. Values are the mean ± S.E.M. from 3 independent experiments run in triplicate. *$p<0.05$ vs. negative siRNA-transfected cells, Student "t" test. H= apoptosis value under hypoxic conditions after subtraction of the apoptosis value detected under normoxic conditions.*

4.9 Inhibition of $P2Y_2$ prevents ischemia/hypoxia-induced apoptosis

To evaluate the role of P2Y receptor subtypes in the induction of apoptosis by the ischemic/hypoxic stress, we used selective $P2Y_2$ and $P2Y_6$ antagonists. AR-C118925, the only known selective, heterocyclic antagonist of the $P2Y_2$ receptor, significantly prevented the appearance of apoptosis by -50.8% ± 2 (Figure 4.10). By contrast, the potent antagonist of $P2Y_6$ nucleotide receptor diisothiocyanate derivative MRS2578 did not affect hypoxia-induced apoptosis in HL-1 cardiomyocytes (Figure 4.10). This pharmacological approach could not be adopted for the $P2Y_4$ receptor, since no selective antagonists are currently available for this receptor subtype. Globally, these pharmacological data support a role for $P2Y_2$, but not $P2Y_6$, in hypoxia-induced apoptosis.

In order to validate these results, we adopted a second approach based on the use of specific siRNAs against the various P2Y receptors. Semi-quantitative real time analysis of $P2Y_2$, and $P2Y_6$ mRNAs after transfection of HL-1 cardiomyocytes with specific siRNAs showed a reduction of corresponding receptor mRNAs of 70%±6.8 and 89%±3.6, for the $P2Y_2$ and $P2Y_6$ receptors, respectively (Figure 4.11A and Figure 4.11B). Semi-quantitative real time analysis could not be performed for the $P2Y_4$ receptor in silenced cells, due to the lack of efficacious oligonucletide primers for this type of analysis. However, standard RT-PCR in cardiomyocytes transfected with $P2Y_4$ siRNA showed a dramatic reduction of $P2Y_4$ amplification product at the expected

447 pb (see arrow in figure 4.11C) with respect to cells transfected with corresponding negative siRNAs.

In line with the pharmacological data of Figure 4.10, silencing of $P2Y_2$ resulted in a marked prevention of apoptosis following ischemic/hypoxic stress (Figure 4.12A). No effect was instead detected after silencing of $P2Y_6$ (Figure 4.12B). Of interest, gene silencing of $P2Y_4$ resulted in a significant increase of cell apoptosis during ischemic/hypoxic stress, thus suggesting a potential protective role for this receptor (Figure 4.12C).

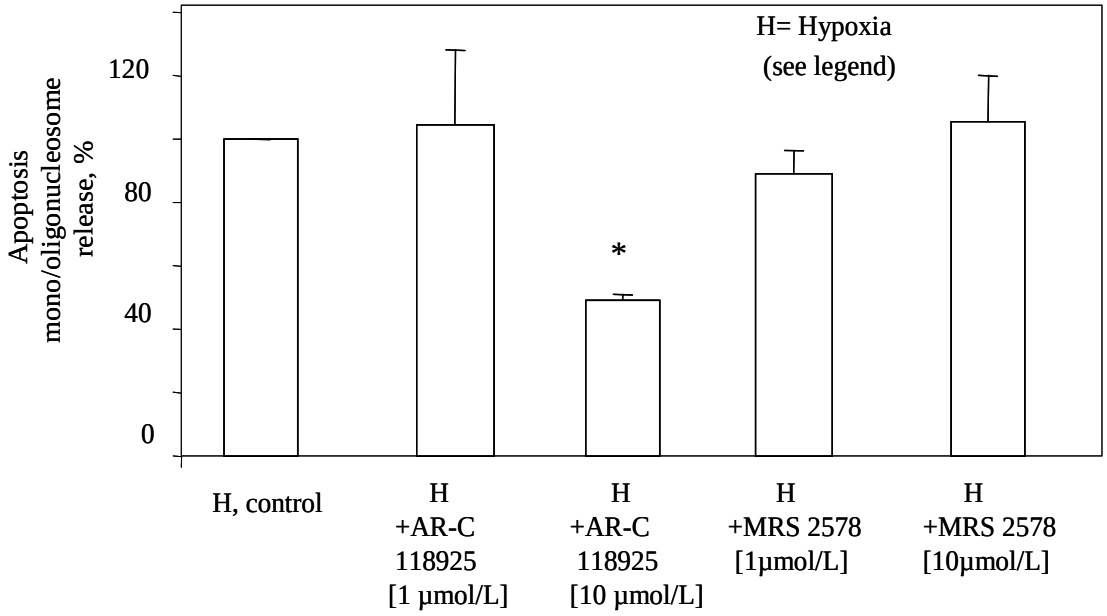


Figure 4.10. Effect of the selective $P2Y_2$ receptor antagonist AR-C118925 and the selective $P2Y_6$ receptor antagonist MRS2578 on induction of cardiomyocyte apoptosis after ischemic hypoxia*. AR-C118925 (1-10 µmol/L) and MRS2578 (1-10µmol/L) were added to cells 1 h before either normoxia or hypoxia. At the end of the 16 h of incubation, apoptosis values were determined in hypoxic cells by ELISA assay and set to 100%. Values are the mean ± S.E.M. from 3 individual experiments run in triplicate, *p<0.05 versus corresponding control values, Student "t" test. H= apoptosis value under hypoxic conditions after subtraction of the apoptosis value detected under normoxic conditions.*

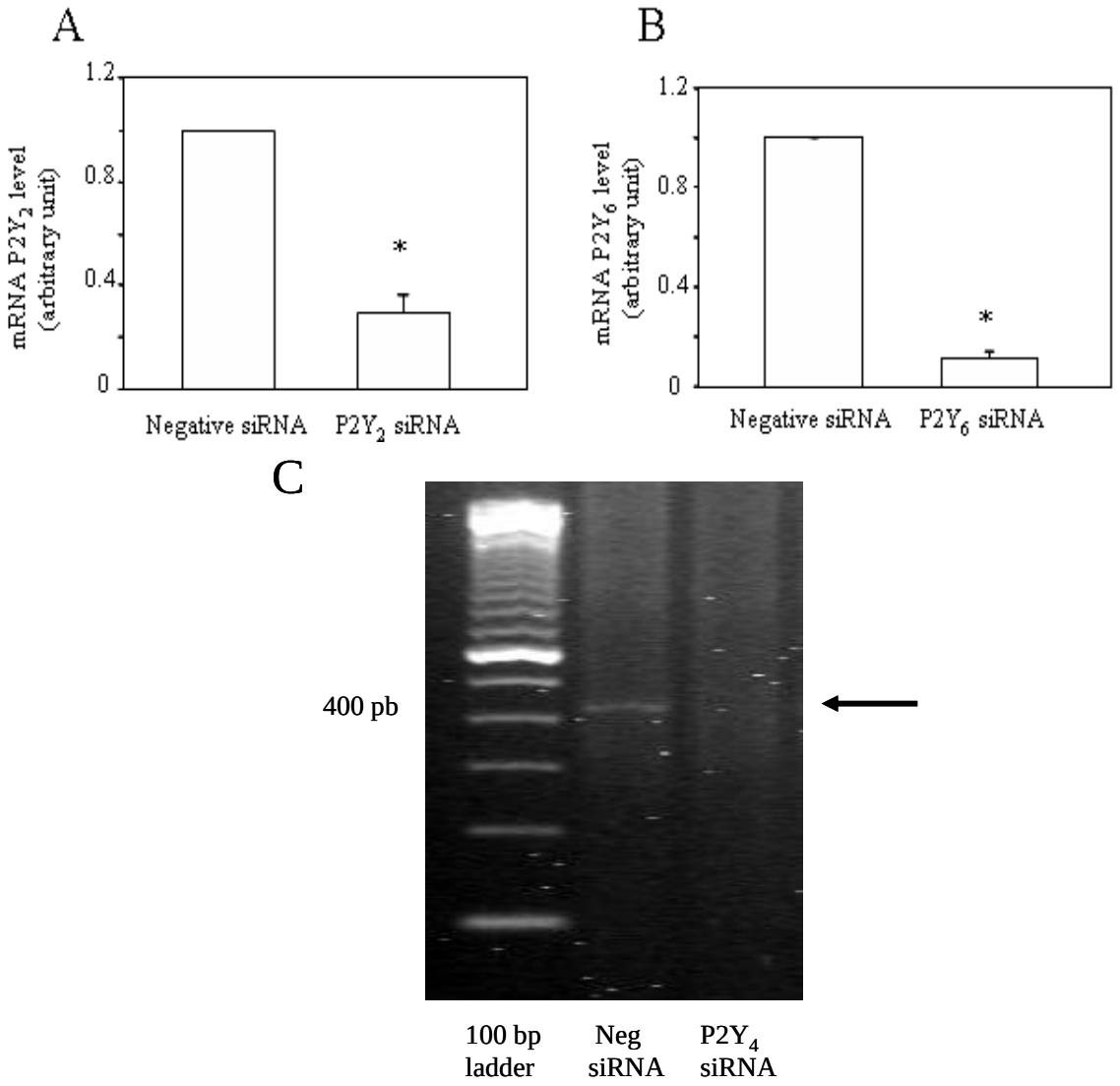


Figure 4.11. Gene silencing. of P2Y$_2$, P2Y$_4$, and P2Y$_6$ A*, mRNA analysis of P2Y$_2$ by quantitative (q) RT-PCR in HL-1 cells transfected with either negative siRNA or with P2Y$_2$ siRNA.* ***B****, mRNA analysis of P2Y$_6$ by qRT-PCR in HL-1 cells transfected with either negative siRNA or with P2Y$_6$ siRNA Values are the means ± S.E.M. of 3 individual experiments run in triplicate.*:p< 0.05 versus control siRNA-negative treated cells, Student "t" test.* ***C****, micrograph showing amplification of P2Y$_4$ mRNA by standard RT-PCR in HL-1 cells transfected with either negative siRNA or with specific P2Y$_4$ siRNA. An amplicon of the expected molecolar weight(see arrow) was detected in control cells; this product was marked reduced after silencing. Shown are results from one experiment. Similar data have been obtain in 3 indipendent experimets analysis of P2Y$_4$.*

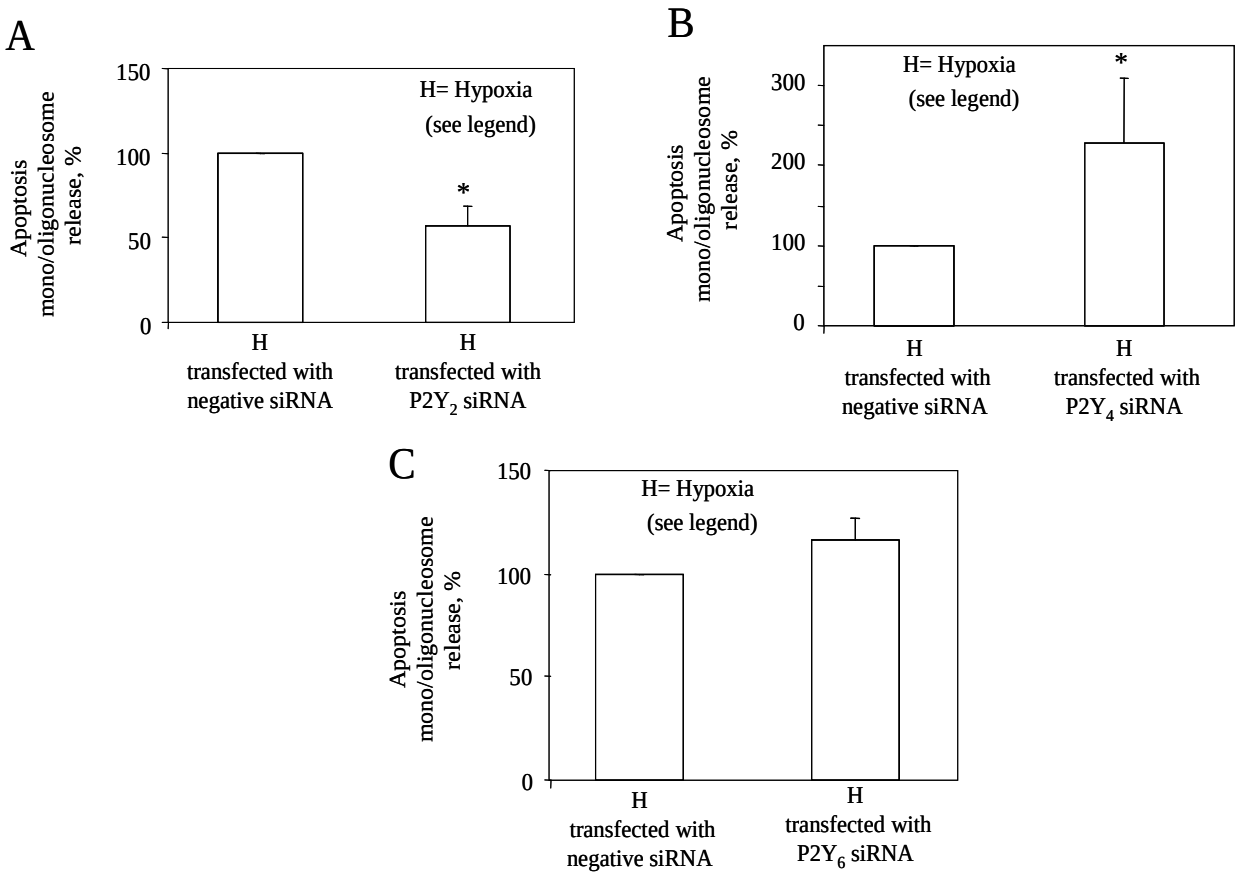


Figure 4.12. Effect of $P2Y_2$, $P2Y_4$, and $P2Y_6$ gene silencing on the induction of cardiomyocyte apoptosis after ischemic hypoxia*. **A**, HL-1 cardiomyocytes transfected with either negative siRNA or with specific $P2Y_2$ siRNA were exposed to normoxia or hypoxia. After 16 h, apoptosis was determined in hypoxic cells and set to 100%. **B**, HL-1 cardiomyocytes transfected with either negative siRNA or with specific $P2Y_4$ siRNA were exposed to normoxia or hypoxia. After 16 h apoptosis was determined in hypoxic cells as described.**C**, HL-1 cardiomyocytes transfected with either negative siRNA or with specific $P2Y_6$ siRNA were exposed to normoxia or hypoxia. After 16 h apoptosis was determined in hypoxic cells as described. Values are the means ± S.E.M. from 3 individual experiments run in triplicate *p<0.05 vs. control siRNA-negative treated cells, Student "t" test. H= apoptosis value under hypoxic conditions after subtraction of the apoptosis value detected under normoxic conditions.*

4.10 Both G proteins and protein kinase C are involved in the cardiomyocyte apoptosis induced by hypoxia and ATP

To shed some light on the post-receptor mechanisms involved in hypoxia and ATP-induced apoptosis of HL-1 cardiomyocytes, we also performed experiments with either the selective Gi protein inhibitor PTX, the pan-G protein inhibitor GDP-β-S, or the protein kinase C inhibitor GF. Both G-protein inhibitors partially but significantly inhibited hypoxia-associated apoptosis, while the protein kinase C inhibitor showed a non statistically significant trend to a reduction (Figure 4.13). When apoptosis was induced by exposure to high ATP concentrations, a highly significant reduction of cell death was observed in the presence of either PTX or GF. No significant effects were induced by these agents on normoxic cells.

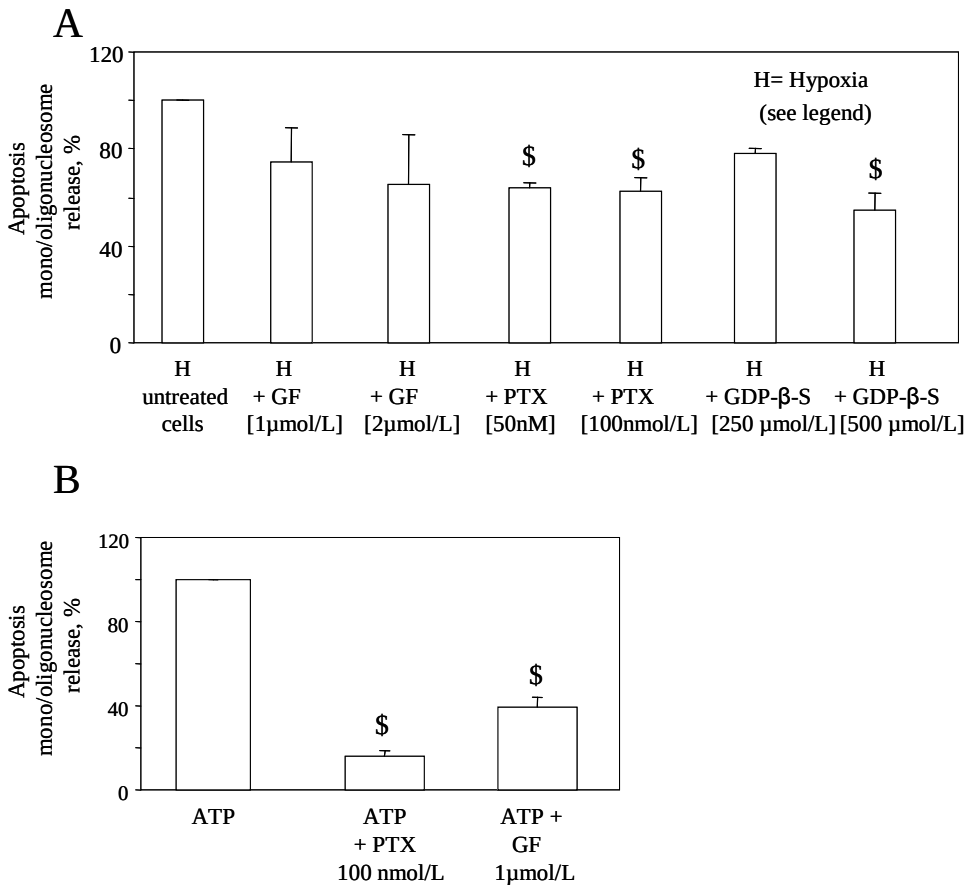


Figure 4.13: Effect of various inhibitors of signalling cascades on cardiomyocyte apoptosis induced by ischemic hypoxia or by ATP. A*, The protein kinase C inhibitor GF (1-2 µmol/L), the Gi protein inhibitor PTX (50-100 nmol/L) and the non specific G-protein inhitor GDP-β-S (250-500 µmol/L) were added to cells 1 h before hypoxia. Parallel samples receiving these same inhibitors were maintained under normoxic conditions. At the end of the incubation, the extent of apoptosis in hypoxic cells was determined by assessing enrichment of mono/oligonucleosome in cells via ELISA assay, and set to 100%. H= apoptosis value under hypoxic conditions after subtraction of the apoptosis value detected under normoxic conditions.**B,** HL-1 cells were treated with ATP at the indicated concentrations. PTX (100 nmol/L) and GF (1µmol/L) were added to cells 1 h before ATP addition (250 µmol/L). At the end of a 24 h incubation, apoptosis was determined by assessing enrichment of mono/oligonucleosome in ATP treated cells via ELISA assay in the absence or presence of the indicated inhibitors and set to 100%. Values are the mean ± S.E.M. from 3 individual experiments, run in triplicate $: p<0.01 versus corresponding ATP control values, Student "t" test.*

5 DISCUSSION

Extracellular nucleotides play important regulatory roles within the cardiovascular system. ATP and UTP are released from either the heart or from other sources during cardiac ischaemia and patients with myocardial infarction have higher plasma levels of both nucleotides (Vassort, 2001; Wihlborg et al., 2006).

Here, we show that ATP and P2 receptors play a key role in hypoxia-induced apoptosis of HL-1 cardiomyocytes, that have been shown to express various P2X and P2Y receptor subtypes (Mazzola et al., 2008).

Specifically, we demonstrate that (i) ischemic/hypoxic stress is associated to rapid ATP release from cultured cardiomyocytes, and, (ii) that distinct P2 receptor subtypes regulate cardiomyocyte death, with both pro- and anti-apoptotic effects. These effect are likely mediated by direct stimulation of P2 receptors by ATP, although we cannot exclude that other concomitantly released nucleotides (e.g., uracil nucleotides, see also below) are also involved. Moreover, by using both pharmacological inhibitors and gene silencing of selected P2 receptor subtypes via small RNA interference, we provide compelling evidence for a specific role of the ligand-gated $P2X_7$ receptor and the G-protein-coupled $P2Y_2$ receptors in hypoxia-induced cardiomyocyte apoptosis. Our data also suggest a protective role for the $P2Y_4$ receptor; conversely, no effects are apparently induced by the co-expressed $P2Y_6$ receptor. Finally, we provide some initial hints involving G-proteins and protein kinase C in the post-receptor mechanisms at the basis of hypoxia- and ATP-induced cardiomyocyte apoptosis.

These data are particularly relevant to the pathogenic mechanisms at the basis of myocardial damage during

hypoxic/ischemia event in patients. Under physiological conditions, in both rat and human heart, as well as in human plasma, extracellular ATP levels have been estimated to be approximately around 20-40 nmol/L (Forrester, 1972; Kuzmin et al., 1998). However, upon hypoxia/ischemia, due to both increased exocytosis from cell's vesicular pool and to release from the cytoplasm (see also below), these levels are markedly increased to reach micromolar concentrations (Dutta et al., 2004). More recently, during myocardial infarction, human venous plasma levels of UTP have been also demonstrated to increase to about 10% of the ATP concentrations (Wihlborg et al., 2006). It has been postulated that, under these conditions, increases of nucleotides release is originally meant at augmenting heart function via a positive inotropic effect mainly exerted through the myocardial $P2Y_2$ receptor (although also $P2Y_4$ and $P2Y_6$ receptors may contribute) (ibidem). However, if heart reperfusion is impaired or totally prevented and nucleotides are not removed from the myocardium, their local concentrations (especially those of ATP) may increase to toxic levels and contribute to myocardial cell death. This hypothesis is consistent with our present data demonstrating that ATP is indeed released from cardiomyocytes very early after an hypoxic/ischemic insult, and that this release is associated to apoptosis induction. In our model, the early release of ATP does not involve cell lysis, since it is not associated to LDH release. Moreover, in line with previous data, ATP release occurs via the maxi-anion channel, which is highly expressed in the heart and rapidly activated by hypoxic stress (Sabirov and Okada, 2005). A causal relationship between ATP release and the

subsequent cardiomyocyte death is confirmed by the demonstration that both non selective P2 receptor blockers, the maxi-anion channel blocker $GdCl_3$ and apyrase (which degrades nucleotide triphosphates into nucleotide monophosphates) were able to inhibit cardiomyocyte apoptosis (Sugimoto et al., 2009). In line with these findings, apyrase expression was increased in pathological samples of patients with ischemic heart disease (Erlinge and Burnstock, 2008; Kittel et al., 2005).

As highlighted above, our data point to differential roles of various P2 receptors in cardiomyocyte viability during hypoxic ischemia. In detail, the pro-apoptotic role of the $P2Y_2$ receptor under hypoxic conditions is in line with previous data demonstrating that this is indeed the most expressed P2 receptor in the heart which undergoes upregulation in the human failing heart (Hou et al., 1999). Moreover, 3 single nucleotide polymorphisms (SNPs) of the human $P2Y_2$ receptor gene have been associated with MI and proposed as genetic markers for MI in Japanese men (Wang et al., 2009). The $P2Y_2$ receptor also promotes a profibrotic response in isolated rat myocardial fibroblasts; moreover, in mice lacking this receptor subtype, extracellular nucleotides are no longer able to increase the expression of the two pro-fibrotic markers plasminogen activator inhibitor type 1 and alpha-smooth muscle actin (Braun et al.). Regarding the $P2X_7$ receptor, despite abundant information on the role of this receptor in induction of apoptosis in macrophages, monocytes, microglia and other cell types (Baraldi et al., 2004), very little is known on its presence and role in MI. While no direct evidence for the presence of $P2X_7$ in rodent heart is available, in dog, this receptor subtype has been

indeed originally cloned from heart cDNA (Roman et al., 2009). Significant levels of $P2X_7$ have been found in human myocardial samples from both normal subjects and patients with chronic heart failure (Banfi et al., 2005). Moreover, a loss-of-function $P2X_7$ polymorphism has been reported in patients with heart failure (Eslick et al., 2009), but the relevance of this finding is unknown. More recently, an indirect involvement of $P2X_7$ in the regulation of cardiomyocyte viability has been suggested by the demonstration that pannexin-1/$P2X_7$ channels mediate the release of cardioprotectants induced by ischemic pre- and post-conditioning (Vessey et al., 2010). Therefore, this is the first time that, based on both pharmacological and gene-silencing data, $P2X_7$ is unequivocally implicated in induction of ischemia-associated cardiomyocyte death.

Our data also highlight a protective role for $P2Y_4$. Previous data have shown that, in cultured hypoxic cardiomyocytes, activation of some P2Y receptors by UTP reduced LDH release (Yitzhaki et al., 2005), prevented intracellular calcium elevation, maintained an increased level of intracellular ATP (Yitzhaki et al., 2006) and preserved mitochondrial activity (Yitzhaki et al., 2007). In line with these findings, in vivo experiments showed that UTP administration reduced infarct size and improved myocardial function (Shainberg et al., 2009). However, the exact P2 receptor involved was not univocally identified in these studies. UTP can activate both $P2Y_2$ and $P2Y_4$ receptors (Abbracchio et al., 2006); the present data indeed suggest, for the first time, that it is the latter that mediates UTP-induced protection. Regarding the $P2Y_6$ receptor subtype, that has been proposed to mediate positive

inotropic effects (Wihlborg et al., 2006), recent papers have indeed involved this receptor in pressure overload-induced cardiac fibrosis (Erlinge and Burnstock, 2008; Nishida et al., 2008). However, our data suggest that, despite playing a role in heart remodeling during chronic heart failure, this receptor is not directly involved in regulation of cardiomyocyte viability during acute hypoxic stress.

Finally, the present data shed some light on the post-receptor mechanisms involved in P2 receptor regulation of cardiomyocyte viability. In our model, inhibition of both G-proteins and PKC resulted in apoptosis reduction (although, for the PKC inhibitor, this effect did not reach statistical significance). These data are in line with our proposal that the G-protein coupled $P2Y_2$ receptor participates to cardiomyocyte death under hypoxic conditions. Regarding the involvement of PKC in cell death, both protective and pro-apoptotic effects have been reported. Whereas activation of PKCδ during reperfusion mediated damage, activation of PKCε before ischaemia protected the heart (Churchill and Mochly-Rosen, 2007). In particular, regulation of PKCδ is critical for cell survival and caspase-mediated cleavage of PKCδ in the nucleus signals an irreversible committment to apoptosis (Reyland, 2007). Regarding the link between P2Y receptors and PKC, $P2Y_2$ is indeed known to couple to the Gq protein that, by activating phospholipase C, promotes the formation of inositol phosphates and diacyl-glycerol, that, can, in turn, activate PKC (Abbracchio et al., 2006). Thus, PCK could be directly recruited by the $P2Y_2$ receptor during ischemic/hypoxic stress and participate to induction of cell death. However, in other cell systems, PKC has

also been reported to mediate $P2Y_2$ receptor desensitization and sequestration (Garrad et al., 1998), which should inhibit cell death. Alternatively, PKC could be recruited by the $P2X_7$ receptor, which also induces cardiomyocyte apoptosis in our model. A direct link between $P2X_7$ and PKC is indeed supported by evidences obtained in other experimental models. In osteoclasts, activation of $P2X_7$ causes isoform-specific translocation of protein kinase C (Armstrong et al., 2009); moreover, in granule neurons, $P2X_7$ receptor is mainly activated in PKC-dependent manner (Ortega et al.). Globally, we demostrated that both $P2Y_2$ and $P2X_7$ contribute to cardiomyocyte apoptosis induction during ischemic/hypoxia. We belive that a pharmacological modulation of this receptor subtypes may have important therapeutic implications and our study set the basis for the development of novel cardioprotective agents that target specific P2 receptor subtypes.

.

6 REFERENCES

Abbracchio, M. P., Boeynaems, J. M., Barnard, E. A., Boyer, J. L., Kennedy, C., Miras-Portugal, M. T., King, B. F., Gachet, C., Jacobson, K. A., Weisman, G. A. et al. *(2003). Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. Trends Pharmacol Sci* ***24****, 52-5.*

Abbracchio, M. P. and Burnstock, G. *(1994). Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther* ***64****, 445-75.*

Abbracchio, M. P. and Burnstock, G. *(1998). Purinergic signalling: pathophysiological roles. Jpn J Pharmacol* ***78****, 113-45.*

Abbracchio, M. P., Burnstock, G., Boeynaems, J. M., Barnard, E. A., Boyer, J. L., Kennedy, C., Knight, G. E., Fumagalli, M., Gachet, C., Jacobson, K. A. et al. *(2006). International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev* ***58****, 281-341.*

Abbracchio, M. P., Ceruti, S., Bolego, C., Puglisi, L., Burnstock, G. and Cattabeni, F. *(1996). Trophic roles of P2 purinoceptors in central nervous system astroglial cells. Ciba Found Symp* ***198****, 142-7; discussion 147-8.*

Amisten, S., Melander, O., Wihlborg, A. K., Berglund, G. and Erlinge, D. *(2007). Increased risk of acute myocardial infarction and elevated levels of C-reactive protein in carriers of the Thr-87 variant of the ATP receptor P2Y11. Eur Heart J* ***28****, 13-8.*

Anversa, P., Cheng, W., Liu, Y., Leri, A., Redaelli, G. and Kajstura, J. *(1998). Apoptosis and myocardial infarction. Basic Res Cardiol* ***93 Suppl 3****, 8-12.*

Armstrong, S., Pereverzev, A., Dixon, S. J. and Sims, S. M. *(2009). Activation of P2X7 receptors causes isoform-specific translocation of protein kinase C in osteoclasts. J Cell Sci* ***122****, 136-44.*

Balogh, J., Wihlborg, A. K., Isackson, H., Joshi, B. V., Jacobson, K. A., Arner, A. and Erlinge, D. *(2005). Phospholipase C and cAMP-dependent positive inotropic effects of ATP in mouse cardiomyocytes via P2Y11-like receptors. J Mol Cell Cardiol* ***39****, 223-30.*

Banfi, C., Ferrario, S., De Vincenti, O., Ceruti, S., Fumagalli, M., Mazzola, A., N, D. A., Volonte, C., Fratto, P., Vitali, E. et al. *(2005). P2 receptors in human heart: upregulation of P2X6 in patients undergoing heart transplantation, interaction with TNFalpha and potential role in myocardial cell death. J Mol Cell Cardiol* ***39****, 929-39.*

Baraldi, P. G., Di Virgilio, F. and Romagnoli, R. *(2004). Agonists and antagonists acting at P2X7 receptor. Curr Top Med Chem* ***4****, 1707-17.*

Berg, J., Lindgren, P., Spiesser, J., Parry, D. and Jonsson, B. *(2007). Cost-effectiveness of clopidogrel in myocardial infarction with ST-segment elevation: a European model based on the CLARITY and COMMIT trials. Clin Ther* ***29****, 1184-202.*

Berger, P. B., Bhatt, D. L., Fuster, V., Steg, P. G., Fox, K. A., Shao, M., Brennan, D. M., Hacke, W., Montalescot, G., Steinhubl, S. R. et al. *Bleeding complications with dual antiplatelet therapy among patients with stable vascular disease or risk factors for vascular disease: results from the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial. Circulation* ***121****, 2575-83.*

Bergfeld, G. R. and Forrester, T. *(1992). Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. Cardiovasc Res* ***26****, 40-7.*

Birk, A. V., Leno, E., Robertson, H. D., Bolotina, V. M. and Szeto, H. H. *(2003). Interaction between ATP and catecholamines in stimulation of platelet aggregation. Am J Physiol Heart Circ Physiol* ***284****, H619-25.*

Boeynaems, J. M., van Giezen, H., Savi, P. and Herbert, J. M. *(2005). P2Y receptor antagonists in thrombosis. Curr Opin Investig Drugs* ***6****, 275-82.*

Bogdanov, Y., Rubino, A. and Burnstock, G. *(1998). Characterisation of subtypes of the P2X and P2Y families of ATP receptors in the foetal human heart. Life Sci* ***62****, 697-703.*

Borna, C., Lazarowski, E., van Heusden, C., Ohlin, H. and Erlinge, D. *(2005). Resistance to aspirin is increased by ST-elevation myocardial infarction and correlates with adenosine diphosphate levels. Thromb J* ***3****, 10.*

Bouchie, J. L., Chen, H. C., Carney, R., Bagot, J. C., Wilden, P. A. and Feener, E. P. *(2000). P2Y receptor regulation of PAI-1 expression in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol* ***20****, 866-73.*

Braun, O. O., Lu, D., Aroonsakool, N. and Insel, P. A. *Uridine triphosphate (UTP) induces profibrotic responses in cardiac fibroblasts by activation of P2Y2 receptors. J Mol Cell Cardiol* ***49****, 362-9.*

Brown, J. and Brown, C. A. *(2002). Evaluation of reactive blue 2 derivatives as selective antagonists for P2Y receptors. Vascul Pharmacol* ***39****, 309-15.*

Burnstock, G. *(1976). Do some nerve cells release more than one transmitter? Neuroscience* ***1****, 239-48.*

Burnstock, G. *(1987). Local control of blood pressure by purines. Blood Vessels* ***24****, 156-60.*

Burnstock, G. *(1997). The past, present and future of purine nucleotides as signalling molecules. Neuropharmacology* ***36****, 1127-39.*

Burnstock, G. *(2006). Purinergic P2 receptors as targets for novel analgesics. Pharmacol Ther* ***110****, 433-54.*

Chambers, J. K., Macdonald, L. E., Sarau, H. M., Ames, R. S., Freeman, K., Foley, J. J., Zhu, Y., McLaughlin, M. M., Murdock, P., McMillan, L. et al. *(2000). A G protein-coupled receptor for UDP-glucose. J Biol Chem* ***275****, 10767-71.*

Churchill, E. N. and Mochly-Rosen, D. *(2007). The roles of PKCdelta and epsilon isoenzymes in the regulation of myocardial ischaemia/reperfusion injury. Biochem Soc Trans* ***35****, 1040-2.*

Ciana, P., Fumagalli, M., Trincavelli, M. L., Verderio, C., Rosa, P., Lecca, D., Ferrario, S., Parravicini, C., Capra, V., Gelosa, P. et al. *(2006). The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. EMBO J* ***25****, 4615-27.*

Clyne, J. D., LaPointe, L. D. and Hume, R. I. *(2002). The role of histidine residues in modulation of the rat P2X(2) purinoceptor by zinc and pH. J Physiol* ***539****, 347-59.*

Communi, D., Gonzalez, N. S., Detheux, M., Brezillon, S., Lannoy, V., Parmentier, M. and Boeynaems, J. M. *(2001). Identification of a novel human ADP receptor coupled to G(i). J Biol Chem* ***276****, 41479-85.*

Communi, D., Robaye, B. and Boeynaems, J. M. *(1999). Pharmacological characterization of the human P2Y11 receptor. Br J Pharmacol* ***128****, 1199-206.*

Costanzi, S., Tikhonova, I. G., Ohno, M., Roh, E. J., Joshi, B. V., Colson, A. O., Houston, D., Maddileti, S., Harden, T. K. and Jacobson, K. A. *(2007). P2Y1 antagonists: combining receptor-based modeling and QSAR for a quantitative prediction of the biological activity based on consensus scoring. J Med Chem* ***50****, 3229-41.*

Danziger, R. S., Raffaeli, S., Moreno-Sanchez, R., Sakai, M., Capogrossi, M. C., Spurgeon, H. A., Hansford, R. G. and Lakatta, E. G. *(1988). Extracellular ATP has a potent effect to enhance cytosolic calcium and contractility in single ventricular myocytes. Cell Calcium* ***9****, 193-9.*

Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J. M., Morelli, A., Torboli, M., Bolognesi, G. and Baricordi, O. R. *(2001). Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood* ***97****, 587-600.*

Di Virgilio, F. and Solini, A. *(2002). P2 receptors: new potential players in atherosclerosis. Br J Pharmacol* ***135****, 831-42.*

Dietrich, H. H., Ellsworth, M. L., Sprague, R. S. and Dacey, R. G., Jr. *(2000). Red blood cell regulation of microvascular tone through adenosine triphosphate. Am J Physiol Heart Circ Physiol* ***278****, H1294-8.*

Dubyak, G. R. *(1991). Signal transduction by P2-purinergic receptors for extracellular ATP. Am J Respir Cell Mol Biol* ***4****, 295-300.*

Dutta, A. K., Korchev, Y. E., Shevchuk, A. I., Hayashi, S., Okada, Y. and Sabirov, R. Z. *(2008). Spatial distribution of maxi-anion channel on cardiomyocytes detected by smart-patch technique. Biophys J* ***94****, 1646-55.*

Dutta, A. K., Sabirov, R. Z., Uramoto, H. and Okada, Y. *(2004). Role of ATP-conductive anion channel in ATP release from neonatal rat cardiomyocytes in ischaemic or hypoxic conditions. J Physiol* ***559****, 799-812.*

Ellsworth, M. L., Forrester, T., Ellis, C. G. and Dietrich, H. H. *(1995). The erythrocyte as a regulator of vascular tone. Am J Physiol* ***269****, H2155-61.*

Ennion, S. J. and Evans, R. J. *(2002). Conserved cysteine residues in the extracellular loop of the human P2X(1) receptor form disulfide bonds and are involved in receptor trafficking to the cell surface. Mol Pharmacol* ***61****, 303-11.*

Erb, L., Garrad, R., Wang, Y., Quinn, T., Turner, J. T. and Weisman, G. A. *(1995). Site-directed mutagenesis of P2U purinoceptors. Positively charged amino acids in transmembrane*

helices 6 and 7 affect agonist potency and specificity. J Biol Chem ***270****, 4185-8.*

Erlinge, D. and Burnstock, G. *(2008). P2 receptors in cardiovascular regulation and disease. Purinergic Signal* ***4****, 1-20.*

Erlinge, D., Harnek, J., van Heusden, C., Olivecrona, G., Jern, S. and Lazarowski, E. *(2005). Uridine triphosphate (UTP) is released during cardiac ischemia. Int J Cardiol* ***100****, 427-33.*

Erlinge, D., Hou, M., Webb, T. E., Barnard, E. A. and Moller, S. *(1998). Phenotype changes of the vascular smooth muscle cell regulate P2 receptor expression as measured by quantitative RT-PCR. Biochem Biophys Res Commun* ***248****, 864-70.*

Eslick, G. D., Thampan, B. V., Nalos, M., McLean, A. S. and Sluyter, R. *(2009). Circulating interleukin-18 concentrations and a loss-of-function P2X7 polymorphism in heart failure. Int J Cardiol* ***137****, 81-3.*

Faxon, D. P. *(2004). Can β-blocker use lower mortality and improve myocardial tissue recovery after acute myocardial infarction? nature reviews cardiology.*

Ferrari, D., Chiozzi, P., Falzoni, S., Dal Susino, M., Melchiorri, L., Baricordi, O. R. and Di Virgilio, F. *(1997). Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. J Immunol* ***159****, 1451-8.*

Firestein, B. L., Xing, M., Hughes, R. J., Corvera, C. U. and Insel, P. A. *(1996). Heterogeneity of P2u- and P2y-purinergic receptor regulation of phospholipases in MDCK cells. Am J Physiol* ***271****, F610-8.*

Forrester, T. *(1972). A quantitative estimation of adenosine triphosphate released from human forearm muscle during sustained exercise. J Physiol* ***221****, 25P-26P.*

Foster, C. J., Prosser, D. M., Agans, J. M., Zhai, Y., Smith, M. D., Lachowicz, J. E., Zhang, F. L., Gustafson, E., Monsma, F. J.,

Jr., Wiekowski, M. T. et al. *(2001). Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. J Clin Invest* ***107****, 1591-8.*

Fraser, J. K., Schreiber, R. E., Zuk, P. A. and Hedrick, M. H. *(2004). Adult stem cell therapy for the heart. Int J Biochem Cell Biol* ***36****, 658-66.*

Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Daly, J. W., Harden, T. K., Jacobson, K. A., Leff, P. and Williams, M. *(1994). Nomenclature and classification of purinoceptors. Pharmacol Rev* ***46****, 143-56.*

Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Dubyak, G. R., Harden, T. K., Jacobson, K. A., Schwabe, U. and Williams, M. *(1997). Towards a revised nomenclature for P1 and P2 receptors. Trends Pharmacol Sci* ***18****, 79-82.*

Froldi, G., Pandolfo, L., Chinellato, A., Ragazzi, E., Caparrotta, L. and Fassina, G. *(1994). Dual effect of ATP and UTP on rat atria: which types of receptors are involved? Naunyn Schmiedebergs Arch Pharmacol* ***349****, 381-6.*

Froldi, G., Ragazzi, E. and Caparrotta, L. *(2001). Do ATP and UTP involve cGMP in positive inotropism on rat atria? Comp Biochem Physiol C Toxicol Pharmacol* ***128****, 265-74.*

Fumagalli, M., Trincavelli, L., Lecca, D., Martini, C., Ciana, P. and Abbracchio, M. P. *(2004). Cloning, pharmacological characterisation and distribution of the rat G-protein-coupled P2Y(13) receptor. Biochem Pharmacol* ***68****, 113-24.*

Gaarder, A., Jonsen, J., Laland, S., Hellem, A. and Owren, P. A. *(1961). Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature* ***192****, 531-2.*

Gachet, C. *(2005). The platelet P2 receptors as molecular targets for old and new antiplatelet drugs. Pharmacol Ther* ***108****, 180-92.*

Garrad, R. C., Otero, M. A., Erb, L., Theiss, P. M., Clarke, L. L., Gonzalez, F. A., Turner, J. T. and Weisman, G. A. *(1998). Structural basis of agonist-induced desensitization and sequestration of the P2Y2 nucleotide receptor. Consequences of truncation of the C terminus. J Biol Chem* ***273****, 29437-44.*

Gu, B., Bendall, L. J. and Wiley, J. S. *(1998). Adenosine triphosphate-induced shedding of CD23 and L-selectin (CD62L) from lymphocytes is mediated by the same receptor but different metalloproteases. Blood* ***92****, 946-51.*

Guo, C., Masin, M., Qureshi, O. S. and Murrell-Lagnado, R. D. *(2007). Evidence for functional P2X4/P2X7 heteromeric receptors. Mol Pharmacol* ***72****, 1447-56.*

Hansson, G. K. *(2005). Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med* ***352****, 1685-95.*

Hou, M., Malmsjo, M., Moller, S., Pantev, E., Bergdahl, A., Zhao, X. H., Sun, X. Y., Hedner, T., Edvinsson, L. and Erlinge, D. *(1999). Increase in cardiac P2X1-and P2Y2-receptor mRNA levels in congestive heart failure. Life Sci* ***65****, 1195-206.*

Hrafnkelsdottir, T., Erlinge, D. and Jern, S. *(2001). Extracellular nucleotides ATP and UTP induce a marked acute release of tissue-type plasminogen activator in vivo in man. Thromb Haemost* ***85****, 875-81.*

Idzko, M., Dichmann, S., Ferrari, D., Di Virgilio, F., la Sala, A., Girolomoni, G., Panther, E. and Norgauer, J. *(2002). Nucleotides induce chemotaxis and actin polymerization in immature but not mature human dendritic cells via activation of pertussis toxin-sensitive P2y receptors. Blood* ***100****, 925-32.*

Jacobson, K. A. and Boeynaems, J. M. *P2Y nucleotide receptors: promise of therapeutic applications. Drug Discov Today* ***15****, 570-8.*

Jacobson, K. A., Jarvis, M. F. and Williams, M. *(2002). Purine and pyrimidine (P2) receptors as drug targets. J Med Chem* ***45****, 4057-93.*

Jiang, Q., Guo, D., Lee, B. X., Van Rhee, A. M., Kim, Y. C., Nicholas, R. A., Schachter, J. B., Harden, T. K. and Jacobson, K. A. *(1997). A mutational analysis of residues essential for ligand recognition at the human P2Y1 receptor. Mol Pharmacol* ***52****, 499-507.*

Kaczmarek, E., Koziak, K., Sevigny, J., Siegel, J. B., Anrather, J., Beaudoin, A. R., Bach, F. H. and Robson, S. C. *(1996). Identification and characterization of CD39/vascular ATP diphosphohydrolase. J Biol Chem* ***271****, 33116-22.*

Kaufmann, A., Musset, B., Limberg, S. H., Renigunta, V., Sus, R., Dalpke, A. H., Heeg, K. M., Robaye, B. and Hanley, P. J. *(2005). "Host tissue damage" signal ATP promotes non-directional migration and negatively regulates toll-like receptor signaling in human monocytes. J Biol Chem* ***280****, 32459-67.*

Kawamura, H., Aswad, F., Minagawa, M., Malone, K., Kaslow, H., Koch-Nolte, F., Schott, W. H., Leiter, E. H. and Dennert, G. *(2005). P2X7 receptor-dependent and -independent T cell death is induced by nicotinamide adenine dinucleotide. J Immunol* ***174****, 1971-9.*

Khakh, B. S., Burnstock, G., Kennedy, C., King, B. F., North, R. A., Seguela, P., Voigt, M. and Humphrey, P. P. *(2001). International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacol Rev* ***53****, 107-18.*

Khakh, B. S. and Henderson, G. *(2000). Modulation of fast synaptic transmission by presynaptic ligand-gated cation channels. J Auton Nerv Syst* ***81****, 110-21.*

Khakh, B. S. and North, R. A. *(2006). P2X receptors as cell-surface ATP sensors in health and disease. Nature* ***442****, 527-32.*

Kim, H., McGrath, B. M. and Silverstone, P. H. *(2005). A review of the possible relevance of inositol and the phosphatidylinositol second messenger system (PI-cycle) to psychiatric disorders--focus on magnetic resonance spectroscopy (MRS) studies. Hum Psychopharmacol* ***20****, 309-26.*

Kim, H. S., Ohno, M., Xu, B., Kim, H. O., Choi, Y., Ji, X. D., Maddileti, S., Marquez, V. E., Harden, T. K. and Jacobson, K. A. *(2003). 2-Substitution of adenine nucleotide analogues containing a bicyclo[3.1.0]hexane ring system locked in a northern conformation: enhanced potency as P2Y1 receptor antagonists. J Med Chem* ***46****, 4974-87.*

Kittel, A., Kiss, A. L., Mullner, N., Matko, I. and Sperlagh, B. *(2005). Expression of NTPDase1 and caveolins in human cardiovascular disease. Histochem Cell Biol* ***124****, 51-9.*

Kloner, R. A. and Jennings, R. B. *(2001). Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 1. Circulation* ***104****, 2981-9.*

Kottgen, M., Loffler, T., Jacobi, C., Nitschke, R., Pavenstadt, H., Schreiber, R., Frische, S., Nielsen, S. and Leipziger, J. *(2003). P2Y6 receptor mediates colonic NaCl secretion via differential activation of cAMP-mediated transport. J Clin Invest* ***111****, 371-9.*

Kroemer, G. and Jaattela, M. *(2005). Lysosomes and autophagy in cell death control. Nat Rev Cancer* ***5****, 886-97.*

Kunapuli, S. P., Dorsam, R. T., Kim, S. and Quinton, T. M. *(2003). Platelet purinergic receptors. Curr Opin Pharmacol* ***3****, 175-80.*

Kuzmin, A. I., Lakomkin, V. L., Kapelko, V. I. and Vassort, G. *(1998). Interstitial ATP level and degradation in control and postmyocardial infarcted rats. Am J Physiol* ***275****, C766-71.*

Lambrecht, G., Braun, K., Damer, M., Ganso, M., Hildebrandt, C., Ullmann, H., Kassack, M. U. and Nickel, P. *(2002). Structure-*

activity relationships of suramin and pyridoxal-5'-phosphate derivatives as P2 receptor antagonists. Curr Pharm Des ***8****, 2371-99.*

Lazarowski, E. R., Boucher, R. C. and Harden, T. K. *(2000). Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. J Biol Chem* ***275****, 31061-8.*

Lazarowski, E. R., Boucher, R. C. and Harden, T. K. *(2003). Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. Mol Pharmacol* ***64****, 785-95.*

Lazarowski, E. R. and Harden, T. K. *(1999). Quantitation of extracellular UTP using a sensitive enzymatic assay. Br J Pharmacol* ***127****, 1272-8.*

Logman, J. F., Heeg, B. M., Herlitz, J. and van Hout, B. A. *Costs and consequences of clopidogrel versus aspirin for secondary prevention of ischaemic events in (high-risk) atherosclerotic patients in Sweden: a lifetime model based on the CAPRIE trial and high-risk CAPRIE subpopulations. Appl Health Econ Health Policy* ***8****, 251-65.*

Malmsjo, M., Adner, M., Harden, T. K., Pendergast, W., Edvinsson, L. and Erlinge, D. *(2000). The stable pyrimidines UDPbetaS and UTPgammaS discriminate between the P2 receptors that mediate vascular contraction and relaxation of the rat mesenteric artery. Br J Pharmacol* ***131****, 51-6.*

Malmsjo, M., Bergdahl, A., Moller, S., Zhao, X. H., Sun, X. Y., Hedner, T., Edvinsson, L. and Erlinge, D. *(1999). Congestive heart failure induces downregulation of P2X1-receptors in resistance arteries. Cardiovasc Res* ***43****, 219-27.*

Mamedova, L. K., Joshi, B. V., Gao, Z. G., von Kugelgen, I. and Jacobson, K. A. *(2004). Diisothiocyanate derivatives as potent,*

*insurmountable antagonists of P2Y6 nucleotide receptors. Biochem Pharmacol **67**, 1763-70.*

***Marchese, A., George, S. R., Kolakowski, L. F., Jr., Lynch, K. R. and O'Dowd, B. F.** (1999). Novel GPCRs and their endogenous ligands: expanding the boundaries of physiology and pharmacology. Trends Pharmacol Sci **20**, 370-5.*

***Marteau, F., Le Poul, E., Communi, D., Labouret, C., Savi, P., Boeynaems, J. M. and Gonzalez, N. S.** (2003). Pharmacological characterization of the human P2Y13 receptor. Mol Pharmacol **64**, 104-12.*

***Mazzola, A., Amoruso, E., Beltrami, E., Lecca, D., Ferrario, S., Cosentino, S., Tremoli, E., Ceruti, S. and Abbracchio, M. P.** (2008). Opposite effects of uracil and adenine nucleotides on the survival of murine cardiomyocytes. J Cell Mol Med **12**, 522-36.*

***Mehta, S. R., Yusuf, S., Peters, R. J., Bertrand, M. E., Lewis, B. S., Natarajan, M. K., Malmberg, K., Rupprecht, H., Zhao, F., Chrolavicius, S. et al.** (2001). Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. Lancet **358**, 527-33.*

***Mei, Q. and Liang, B. T.** (2001). P2 purinergic receptor activation enhances cardiac contractility in isolated rat and mouse hearts. Am J Physiol Heart Circ Physiol **281**, H334-41.*

***Morales-Ramos, A. I., Mecom, J. S., Kiesow, T. J., Graybill, T. L., Brown, G. D., Aiyar, N. V., Davenport, E. A., Kallal, L. A., Knapp-Reed, B. A., Li, P. et al.** (2008). Tetrahydro-4-quinolinamines identified as novel P2Y(1) receptor antagonists. Bioorg Med Chem Lett **18**, 6222-6.*

***Morris, J. B., Pham, T. M., Kenney, B., Sheppard, K. E. and Woodcock, E. A.** (2004). UTP transactivates epidermal growth factor receptors and promotes cardiomyocyte hypertrophy despite inhibiting transcription of the hypertrophic marker gene, atrial natriuretic peptide. J Biol Chem **279**, 8740-6.*

Muller, C. E. *(2002). P2-pyrimidinergic receptors and their ligands. Curr Pharm Des* ***8****, 2353-69.*

Newbolt, A., Stoop, R., Virginio, C., Surprenant, A., North, R. A., Buell, G. and Rassendren, F. *(1998). Membrane topology of an ATP-gated ion channel (P2X receptor). J Biol Chem* ***273****, 15177-82.*

Nishida, M., Sato, Y., Uemura, A., Narita, Y., Tozaki-Saitoh, H., Nakaya, M., Ide, T., Suzuki, K., Inoue, K., Nagao, T. et al. *(2008). P2Y6 receptor-Galpha12/13 signalling in cardiomyocytes triggers pressure overload-induced cardiac fibrosis. EMBO J* ***27****, 3104-15.*

North, R. A. *(2002). Molecular physiology of P2X receptors. Physiol Rev* ***82****, 1013-67.*

Olivecrona, G. K., Gotberg, M., Harnek, J., Wang, L., Jacobson, K. A. and Erlinge, D. *(2004). Coronary artery reperfusion: The ADP receptor P2Y(1) mediates early reactive hyperemia in vivo in pigs. Purinergic Signal* ***1****, 59-65.*

Ortega, F., Perez-Sen, R., Morente, V., Delicado, E. G. and Miras-Portugal, M. T. *P2X7, NMDA and BDNF receptors converge on GSK3 phosphorylation and cooperate to promote survival in cerebellar granule neurons. Cell Mol Life Sci* ***67****, 1723-33.*

Palmer, R. K., Boyer, J. L., Schachter, J. B., Nicholas, R. A. and Harden, T. K. *(1998). Agonist action of adenosine triphosphates at the human P2Y1 receptor. Mol Pharmacol* ***54****, 1118-23.*

Pendergast, W., Yerxa, B. R., Douglass, J. G., 3rd, Shaver, S. R., Dougherty, R. W., Redick, C. C., Sims, I. F. and Rideout, J. L. *(2001). Synthesis and P2Y receptor activity of a series of uridine dinucleoside 5'-polyphosphates. Bioorg Med Chem Lett* ***11****, 157-60.*

Peter, M. E. and Krammer, P. H. *(2003). The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ* ***10****, 26-35.*

Pfefferkorn, J. A., Choi, C., Winters, T., Kennedy, R., Chi, L., Perrin, L. A., Lu, G., Ping, Y. W., McClanahan, T., Schroeder, R. et al. *(2008). P2Y1 receptor antagonists as novel antithrombotic agents. Bioorg Med Chem Lett* ***18****, 3338-43.*

Podrasky, E., Xu, D. and Liang, B. T. *(1997). A novel phospholipase C- and cAMP-independent positive inotropic mechanism via a P2 purinoceptor. Am J Physiol* ***273****, H2380-7.*

Ralevic, V. and Burnstock, G. *(1998). Receptors for purines and pyrimidines. Pharmacol Rev* ***50****, 413-92.*

Rasi, G., DiVirgilio, D., Mutchnick, M. G., Colella, F., Sinibaldi-Vallebona, P., Pierimarchi, P., Valli, B. and Garaci, E. *(1996). Combination thymosin alpha 1 and lymphoblastoid interferon treatment in chronic hepatitis C. Gut* ***39****, 679-83.*

Reyland, M. E. *(2007). Protein kinase Cdelta and apoptosis. Biochem Soc Trans* ***35****, 1001-4.*

Roman, S., Cusdin, F. S., Fonfria, E., Goodwin, J. A., Reeves, J., Lappin, S. C., Chambers, L., Walter, D. S., Clay, W. C. and Michel, A. D. *(2009). Cloning and pharmacological characterization of the dog P2X7 receptor. Br J Pharmacol* ***158****, 1513-26.*

Sabirov, R. Z. and Okada, Y. *(2005). ATP release via anion channels. Purinergic Signal* ***1****, 311-28.*

Saini, H. K., Elimban, V. and Dhalla, N. S. *(2005). Attenuation of extracellular ATP response in cardiomyocytes isolated from hearts subjected to ischemia-reperfusion. Am J Physiol Heart Circ Physiol* ***289****, H614-23.*

Scamps, F. and Vassort, G. *(1994). Pharmacological profile of the ATP-mediated increase in L-type calcium current amplitude*

*and activation of a non-specific cationic current in rat ventricular cells. Br J Pharmacol **113**, 982-6.*

***Schmid-Antomarchi, H., Schmid-Alliana, A., Romey, G., Ventura, M. A., Breittmayer, V., Millet, M. A., Husson, H., Moghrabi, B., Lazdunski, M. and Rossi, B.** (1997). Extracellular ATP and UTP control the generation of reactive oxygen intermediates in human macrophages through the opening of a charybdotoxin-sensitive Ca2+-dependent K+ channel. J Immunol **159**, 6209-15.*

***Scorrano, L., Ashiya, M., Buttle, K., Weiler, S., Oakes, S. A., Mannella, C. A. and Korsmeyer, S. J.** (2002). A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell **2**, 55-67.*

***Serebruany, V. L.** The TRITON versus PLATO trials: differences beyond platelet inhibition. Thromb Haemost **103**, 259-61.*

***Seye, C. I., Yu, N., Jain, R., Kong, Q., Minor, T., Newton, J., Erb, L., Gonzalez, F. A. and Weisman, G. A.** (2003). The P2Y2 nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells. J Biol Chem **278**, 24960-5.*

***Shainberg, A., Yitzhaki, S., Golan, O., Jacobson, K. A. and Hochhauser, E.** (2009). Involvement of UTP in protection of cardiomyocytes from hypoxic stress. Can J Physiol Pharmacol **87**, 287-99.*

***Shaver, S. R., Rideout, J. L., Pendergast, W., Douglass, J. G., Brown, E. G., Boyer, J. L., Patel, R. I., Redick, C. C., Jones, A. C., Picher, M. et al.** (2005). Structure-activity relationships of dinucleotides: Potent and selective agonists of P2Y receptors. Purinergic Signal **1**, 183-91.*

***Simon, J., Filippov, A. K., Goransson, S., Wong, Y. H., Frelin, C., Michel, A. D., Brown, D. A. and Barnard, E. A.** (2002). Characterization and channel coupling of the P2Y(12) nucleotide*

receptor of brain capillary endothelial cells. J Biol Chem ***277****, 31390-400.*

Springthorpe, B., Bailey, A., Barton, P., Birkinshaw, T. N., Bonnert, R. V., Brown, R. C., Chapman, D., Dixon, J., Guile, S. D., Humphries, R. G. et al. *(2007). From ATP to AZD6140: the discovery of an orally active reversible P2Y12 receptor antagonist for the prevention of thrombosis. Bioorg Med Chem Lett* ***17****, 6013-8.*

Stoop, R., Thomas, S., Rassendren, F., Kawashima, E., Buell, G., Surprenant, A. and North, R. A. *(1999). Contribution of individual subunits to the multimeric P2X(2) receptor: estimates based on methanethiosulfonate block at T336C. Mol Pharmacol* ***56****, 973-81.*

Sugimoto, S., Lin, X., Lai, J., Okazaki, M., Das, N. A., Li, W., Krupnick, A. S., Chen, R., Jeong, S. S., Patterson, G. A. et al. *(2009). Apyrase treatment prevents ischemia-reperfusion injury in rat lung isografts. J Thorac Cardiovasc Surg* ***138****, 752-9.*

Suzuki, T., Hide, I., Ido, K., Kohsaka, S., Inoue, K. and Nakata, Y. *(2004). Production and release of neuroprotective tumor necrosis factor by P2X7 receptor-activated microglia. J Neurosci* ***24****, 1-7.*

Takagi, H., Matsui, Y., Hirotani, S., Sakoda, H., Asano, T. and Sadoshima, J. *(2007). AMPK mediates autophagy during myocardial ischemia in vivo. Autophagy* ***3****, 405-7.*

Tantry, U. S., Bliden, K. P. and Gurbel, P. A. *(2007). Azd6140. Expert Opin Investig Drugs* ***16****, 225-9.*

Todorov, L. D., Mihaylova-Todorova, S., Westfall, T. D., Sneddon, P., Kennedy, C., Bjur, R. A. and Westfall, D. P. *(1997). Neuronal release of soluble nucleotidases and their role in neurotransmitter inactivation. Nature* ***387****, 76-9.*

Torres, G. E., Egan, T. M. and Voigt, M. M. *(1998). Topological analysis of the ATP-gated ionotropic [correction of ionotrophic] P2X2 receptor subunit. FEBS Lett* ***425****, 19-23.*

Vakkila, J. and Lotze, M. T. *(2004). Inflammation and necrosis promote tumour growth. Nat Rev Immunol* ***4****, 641-8.*

Vassort, G. *(2001). Adenosine 5'-triphosphate: a P2-purinergic agonist in the myocardium. Physiol Rev* ***81****, 767-806.*

Vessey, D. A., Li, L. and Kelley, M. *Pannexin-I/P2X 7 purinergic receptor channels mediate the release of cardioprotectants induced by ischemic pre- and postconditioning. J Cardiovasc Pharmacol Ther* ***15****, 190-5.*

Vial, C., Roberts, J. A. and Evans, R. J. *(2004). Molecular properties of ATP-gated P2X receptor ion channels. Trends Pharmacol Sci* ***25****, 487-93.*

Wallentin, L. *(2009). P2Y(12) inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. Eur Heart J* ***30****, 1964-77.*

Wang, L., Karlsson, L., Moses, S., Hultgardh-Nilsson, A., Andersson, M., Borna, C., Gudbjartsson, T., Jern, S. and Erlinge, D. *(2002). P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. J Cardiovasc Pharmacol* ***40****, 841-53.*

Wang, L., Olivecrona, G., Gotberg, M., Olsson, M. L., Winzell, M. S. and Erlinge, D. *(2005). ADP acting on P2Y13 receptors is a negative feedback pathway for ATP release from human red blood cells. Circ Res* ***96****, 189-96.*

Wang, Z. X., Nakayama, T., Sato, N., Izumi, Y., Kasamaki, Y., Ohta, M., Soma, M., Aoi, N., Matsumoto, K., Ozawa, Y. et al. *(2009). Association of the purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) gene with myocardial infarction in Japanese men. Circ J* ***73****, 2322-9.*

Whelan, R. S., Kaplinskiy, V. and Kitsis, R. N. *Cell death in the pathogenesis of heart disease: mechanisms and significance. Annu Rev Physiol* ***72****, 19-44.*

White, P. J., Webb, T. E. and Boarder, M. R. *(2003). Characterization of a Ca2+ response to both UTP and ATP at human P2Y11 receptors: evidence for agonist-specific signaling. Mol Pharmacol* ***63****, 1356-63.*

White, S. M., Constantin, P. E. and Claycomb, W. C. *(2004). Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. Am J Physiol Heart Circ Physiol* ***286****, H823-9.*

Wihlborg, A. K., Balogh, J., Wang, L., Borna, C., Dou, Y., Joshi, B. V., Lazarowski, E., Jacobson, K. A., Arner, A. and Erlinge, D. *(2006). Positive inotropic effects by uridine triphosphate (UTP) and uridine diphosphate (UDP) via P2Y2 and P2Y6 receptors on cardiomyocytes and release of UTP in man during myocardial infarction. Circ Res* ***98****, 970-6.*

Wihlborg, A. K., Wang, L., Braun, O. O., Eyjolfsson, A., Gustafsson, R., Gudbjartsson, T. and Erlinge, D. *(2004). ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. Arterioscler Thromb Vasc Biol* ***24****, 1810-5.*

Wildman, S. S., Unwin, R. J. and King, B. F. *(2003). Extended pharmacological profiles of rat P2Y2 and rat P2Y4 receptors and their sensitivity to extracellular H+ and Zn2+ ions. Br J Pharmacol* ***140****, 1177-86.*

Yang, A., Sonin, D., Jones, L., Barry, W. H. and Liang, B. T. *(2004). A beneficial role of cardiac P2X4 receptors in heart failure: rescue of the calsequestrin overexpression model of cardiomyopathy. Am J Physiol Heart Circ Physiol* ***287****, H1096-103.*

Yegutkin, G., Bodin, P. and Burnstock, G. *(2000). Effect of shear stress on the release of soluble ecto-enzymes ATPase and 5'-*

nucleotidase along with endogenous ATP from vascular endothelial cells. Br J Pharmacol ***129****, 921-6.*

Yerxa, B. R., Sabater, J. R., Davis, C. W., Stutts, M. J., Lang-Furr, M., Picher, M., Jones, A. C., Cowlen, M., Dougherty, R., Boyer, J. et al. *(2002). Pharmacology of INS37217 [P(1)-(uridine 5')-P(4)- (2'-deoxycytidine 5')tetraphosphate, tetrasodium salt], a next-generation P2Y(2) receptor agonist for the treatment of cystic fibrosis. J Pharmacol Exp Ther* ***302****, 871-80.*

Yitzhaki, S., Hochhauser, E., Porat, E. and Shainberg, A. *(2007). Uridine-5'-triphosphate (UTP) maintains cardiac mitochondrial function following chemical and hypoxic stress. J Mol Cell Cardiol* ***43****, 653-62.*

Yitzhaki, S., Shainberg, A., Cheporko, Y., Vidne, B. A., Sagie, A., Jacobson, K. A. and Hochhauser, E. *(2006). Uridine-5'-triphosphate (UTP) reduces infarct size and improves rat heart function after myocardial infarct. Biochem Pharmacol* ***72****, 949-55.*

Yitzhaki, S., Shneyvays, V., Jacobson, K. A. and Shainberg, A. *(2005). Involvement of uracil nucleotides in protection of cardiomyocytes from hypoxic stress. Biochem Pharmacol* ***69****, 1215-23.*

Zheng, J. S., Boluyt, M. O., Long, X., O'Neill, L., Lakatta, E. G. and Crow, M. T. *(1996). Extracellular ATP inhibits adrenergic agonist-induced hypertrophy of neonatal cardiac myocytes. Circ Res* ***78****, 525-35.*

Zheng, J. S., Boluyt, M. O., O'Neill, L., Crow, M. T. and Lakatta, E. G. *(1994). Extracellular ATP induces immediate-early gene expression but not cellular hypertrophy in neonatal cardiac myocytes. Circ Res* ***74****, 1034-41.*

Zimmermann, H. *(1999). Two novel families of ectonucleotidases: molecular structures, catalytic properties and a search for function. Trends Pharmacol Sci* ***20****, 231-6.*

Zimmermann, H. *(2000). Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch Pharmacol* ***362****, 299-309.*

Abbreviations

ACE	angiotensin-converting enzyme
AMI	acute myocardial infarction
CRP	C-reactive protein
CVD	cardiovascular diseases
cysLTs	cysteinyl-leukotrienes
DMEM	Dulbecco's Modified Eagle Medium
ER	endoplasmic reticulum
GPCR	G protein-coupled receptor
IL	interleukin
IUPHAR	International Union of Pharmacology
LDH	lactate dehydrogenase
MI	myocardial infarction
MPTP	mitochondrial permeability trasition pore
NA	noradrenaline
NO	nitric oxide
NTPD	ectonucleotidase
SNPs	single nucleotide polymorphisms
tPA	tissue-type plasminogen activator
VSMC	vascular smooth muscle cells
VCAM-1	vascular cell adhesion molecule-1
2MeSADP	2-methylthioadenosine 5'-diphosphate
PAI-1	plasminogen activator inhibitor
RB2	Reactive blue 2