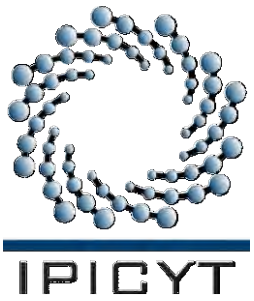

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

Molecular and Electrophysiological Characterization of P2X Receptors in Human Monocytes

Tesis que presenta

Cintya del Refugio López López

Para obtener el grado de

Doctor en Ciencias en Biología Molecular

Codirectores de la Tesis:

Dr. Carlos Barajas López

Dra. Diana Patricia Portales Pérez

San Luis Potosí, S.L.P., Octubre de 2016

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Constancia de aprobación de la tesis

La tesis ***"Molecular and Electrophysiological Characterization of P2X Receptors in Human Monocytes"*** presentada para obtener el Grado de Doctora en Ciencias en Biología Molecular fue elaborada por **Cintya del Refugio López López** y aprobada el **veintiocho de octubre del dos mil dieciséis** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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DOCTORA EN CIENCIAS EN BIOLOGÍA MOLECULAR

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Cintya del Refugio López López

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Molecular and Electrophysiological Characterization of P2X Receptors in Human Monocytes

que se desarrolló bajo la dirección de

Dr. Carlos Barajas López

El Jurado, después de deliberar, determinó

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Dedicatorias

A mis padres María del Refugio López Villa y Jesús López Escobedo.

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Contenido

Lista de Figuras y Tablas

Resumen

Caracterización electrofisiológica y molecular de receptores P2X en monocitos humanos.

Usando técnicas de electrofisiología y biología molecular se analizó la expresión de los receptores P2X en monocitos humanos. Se encontró el mRNA de la subunidad P2X1 y su variante de splicing llamada P2X1*del*, así como del receptor P2X4 en un alto porcentaje de monocitos, en cambio el receptor P2X7 se encontró en un porcentaje mínimo. El análisis en células únicas y electrofisiológico sugiere la expresión heterogénea de subunidades P2X en los monocitos. Las corrientes mediadas por los canales P2X1 (EC_{50}=1.9±0.8 μM) Y P2X1*del* (EC_{50} >1000 μM) expresados en ovocitos de *Xenopus laevis* presentaron diferente cinética y sensibilidad al ATP. Ambas corrientes mediadas por estos receptores mostraron incremento en la amplitud de la corriente bajo un estimulo prolongado con altas concentraciones de ATP. La activación de receptores P2X1 nativos en monocitos humanos mostró una EC_{50}=6.3±0.2 μM. Estas corrientes presentaron características similares a las observadas cuando se expreso a los receptores P2X1 y P2X1*del* en ovocitos. La co-expresión del P2X1 y P2X1*del* mostro una EC_{50}=4.5±1.34 μM y propiedades diferentes a las observadas en ovocitos que expresaron solo a uno de los receptores. Nuestro estudio muestra por primera vez la expresión heterogénea de los receptores P2X y su distribución entre las sub-poblaciones de monocitos humanos, la formación de canales funcionales al expresar al receptor P2X1*del*, y el incremento en la amplitud de corriente en los receptores P2X1y P2X1*del* homoméricos cuando se estimulan con concentraciones altas de ATP por tiempos prolongados, de manera similar a los canales P2X7, P2X2 y P2X4.

Palabras clave: Expresión heteróloga, receptores P2X, células sanguíneas, patch clamp.

Abstract

Molecular and Electrophysiological Characterization of P2X Receptors in Human Monocytes

In order to characterize the general properties and the presence of P2X receptors in single human monocytes we used RT-PCR, flow cytometry, patch-clamp and the two-electrode voltage-clamp techniques. A high percentage of monocytes expressed the canonical P2X1, its splicing variant P2X1*del* and P2X4 mRNAs. P2X7 transcript was found in a minimal fraction of cells. P2X1 receptor immunoreactivity was observed in most monocytes but lower immunoreactivity was found for P2X4 and P2X7. Our data suggest heterogeneous expression of P2X receptors in monocytes and is supported by the electrophysiological data obtained. Currents mediated by P2X1 (EC_{50}=1.9±0.8 μM) and P2X1*del* (EC_{50} >1000 μM) channels, expressed in *Xenopus leavis* oocytes, have different ATP sensitivity and kinetics. Both currents mediated by P2X1 and P2X1*del* channels kept increasing during the continuous presence of high ATP concentrations. Currents mediated by the native P2X1 receptors in human monocytes showed an EC_{50}=6.3±0.2 μM. Currents have kinetics that resemble those observed for P2X1 and P2X1*del* receptors in oocytes. Co-expression of both receptors showed an EC_{50}= 4.65±1.34 μM and different properties to the observed for each receptor expressed alone. This study is the first to demonstrate heterogeneous expression of P2X receptors in human monocytes and their distribution among monocyte subpopulations classified by their expression of CD14 and CD16. Our study is the first in demonstrate the expression of P2X1 receptor and its splicing variant P2X1*del* in most human monocytes. We also, for the first time, described functional homomeric P2X1*del* channels and showed that currents mediated by P2X1 or P2X1*del* increased in amplitude when activated with high ATP concentrations in a similar fashion to those channels that increase their conductance under similar conditions, such as P2X7, P2X2, and P2X4 channels.

Keywords: heterologous expression, P2X receptors, blood cells, patch clamp.

1. INTRODUCTION

The immune system is a defense complex integrated by cells, lymphoid organs, humoral proteins, and cytokines, whose more important function is to protect the host from different threats in the environment. In our environment there are many microorganisms that could enter into our skin and mucous membranes causing infections. The ability of these organisms to cause disease is the result of the virulence factors that the microorganism posses and the integrity of the host defence mechanisms to fight the infection. If the immune system function of the host is compromised it can result in severe infections, tumours, immunodeficiency, autoimmune diseases or allergies (Parkin and Cohen 2001).

The immune system is capable of providing an extensive variety of responses depending on the antigen that causes its activation. A successful host defense is based on the recognition of microbial structures, the innate immune system recognize molecular patterns that are common among different microorganisms but are not present in the host, the adaptive immune system responses are mediated by the production of molecules with high specificity for the antigen causing its production (Chaplin 2006).

The innate immune system activation is the first and the faster line of response to eliminate invading pathogens. It is composed by molecules like antimicrobial peptides and opsonins, and specialized cells known as phagocytes. Phagocytes (monocytes, macrophages, neutrophiles) are cells that could ingest and break

down bacteria and other cells; synthesize inflammatory mediators that can eliminate microorganisms like bacteria, parasites, and viruses; induce the activation of other cell types; and to wipe toxic compounds produced by the metabolism. However, its long time activation could result in inflammation and tissue damage, and the appearance of inflammatory diseases, like atherosclerosis or rheumatoid arthritis (Auffray, Sieweke et al. 2009).

The adaptive response is responsible of eradicate microorganisms that cannot be eliminated by the innate system, this response follows to the activation of the innate system and is mediated by cells able to produce molecules to neutralize specific pathogens; these cells proliferate until their numbers are enough to eliminate the microorganism causing their activation. Cells activated to neutralize any specific pathogen are preserved, giving to this arm of the immune system memory to induce a fast response to pathogens that are in contact with the host for a second time. An effective acquired immune reaction includes two main groups of cells, lymphocytes and antigen presenting cells. Lymphocytes (B cells and T cells) produce and exhibit cell surface receptors that bind antigens and thus mediate the defining attributes of the adaptive immune response: specific response, the ability to respond to several antigens, memory and the property to distinguish among self and non self molecules. Both immune responses collaborate in an active red to effectively protect the host in its environment (Chaplin 2006).

1.1 MONOCYTES

Monocytes are leukocytes from the innate immune system circulating in the blood that play an important role during the inflammatory process and in keeping the homeostasis (Auffray, Sieweke et al. 2009). In humans, monocytes represent 10% of the nucleated cells in the blood and their most recognized function is as a systemic reservoir for the replacement of tissue macrophages (van Furth and Cohn 1968), but also monocytes participate in the elimination of apoptotic cells and toxic compounds as well as in the activation of adaptive immune cells. Monocytes express receptors that allow them to recognize a diverse number of microorganisms, lipid molecules and cells undergoing cell death. Stimulated monocytes can produce large amounts of effector molecules like Reactive Oxygen Species, complement factors, prostaglandins, cytokines and proteolytic enzymes involved in the defense against pathogens and in the pathogenesis of many inflammatory diseases (Serbina, Jia et al. 2008).

Human monocytes are divided into subsets on the basis of surface CD14 (LPS co-receptor) and CD16 (FcγRIII) expression, the $CD14^{+}/CD16^{-}$ (classical) and the $CD14^{+}/CD16^{+}$ (non-classical) monocytes (Passlick, Flieger et al. 1989). Pro-inflammatory CD16+ monocytes consist of two different populations whose expression of chemokine receptors is different (Ancuta, Rao et al. 2003). The intermediate population of monocytes express CD16 and CD14, but CD14 level is lower than the classic monocytes (Ziegler-Heitbrock 2000). Classical monocytes are the biggest subset in human blood and are more phagocytic than non-classical monocytes (Strauss-Ayali, Conrad et al. 2007; Cros, Cagnard et al. 2010);

furthermore these cells have high antimicrobial function. Intermediate monocytes express HLA and CD74 in higher levels, which makes them antigen presenter cells. The intermediate subset produce high levels of Reactive Oxygen Species, IL-1β and TNFα, the expression of chemokine receptors is upregulated and possess proangiogenic capability due to their expression of VEGFR (Serbina, Jia et al. 2008; Wong, Tai et al. 2011; Heine, Ortiz et al. 2012). Non-classical monocytes are called pro-inflammatory and constitute 20% of the blood circulating monocytes, they are responsible for the production of inflammatory cytokines and are preferentially mobilized during inflammation, these monocytes circulate through and watch over the circulatory system. (Strauss-Ayali, Conrad et al. 2007; Auffray, Sieweke et al. 2009; Wong, Yeap et al. 2012). It has been proposed that monocyte subpopulations are different stages in the monocyte to macrophage differentiation. In the bone marrow monocytes are in the classic form (CD14+ CD16-), they are released into the blood where them can occupy inflamed tissues and become macrophages or dendritic cells, but also, these cells can stay in the blood and turn into intermediate monocytes (CD14+CD16+). The intermediate monocytes can evolve in non classic monocytes (CD14+CD16++) in blood, and both, intermediate and non-classic can occupy the host tissues to replace the macrophage cell population on the site (Ziegler-Heitbrock 2007; Heine, Ortiz et al. 2012).

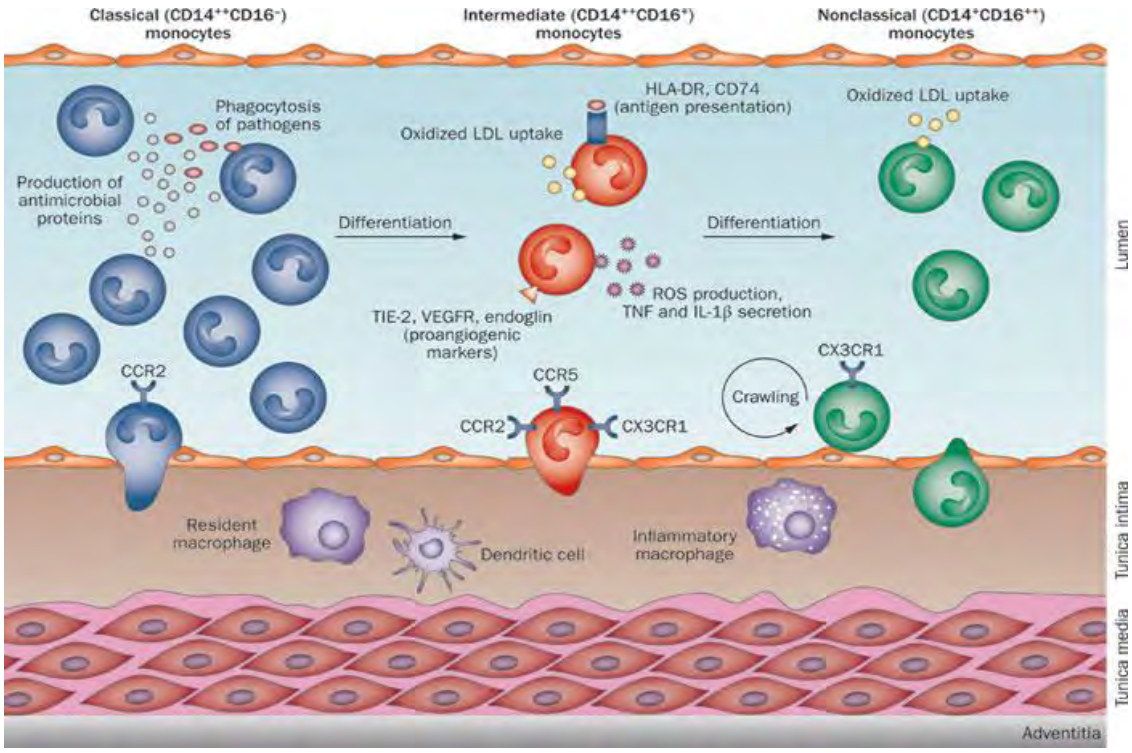

Figure 1. Monocyte subsets and their potential functional roles and differentiation. Taken from: Heine G.H. et al. Nat Rev. Nephrol 8, 362-369 (2012).

1.2 PURINERGIC SIGNALLING IN THE IMMUNE SYSTEM

Adenosine triphosphate (ATP) is the principal supply of energy for the cells; it is present at millimolar concentrations in the cytoplasmic milieu (McCleskey and Gold 1999). Various immune cells consider extracellular ATP as a danger signal (Corriden and Insel 2010). Apoptotic, necrotic or cells under stress situations like mechanical stimulation (Homolya, Steinberg et al. 2000), hypoxia or under invasion of pathogens (McNamara, Khong et al. 2001; Seror, Melki et al. 2011) can release ATP to the extracellular space. Once in the extracellular space, ATP activates receptors such as P2X (ionic channels) and P2Y (G-coupled protein receptors)

expressed in immune cells and the resulting signaling to the extracellular nucleotide is mediated by the activation of both receptor families (Abbracchio and Burnstock 1994; Di Virgilio, Chiozzi et al. 2001). In lymphocytes, ATP is capable of amplifying the T cell receptor mediated signaling, in monocytes and macrophages ATP promotes the activation of the inflammasome (Junger 2011; Ayna, Krysko et al. 2012; Asgari, Le Friec et al. 2013), and the release of interleukins that participate in the inflammatory process. ATP can be released through different mechanisms including exocytosis of secretory granules, vesicular transport, pannexins and connexins and the activation of membrane channels like ATP-binding cassette transporters (Abraham, Prat et al. 1993; Bodin and Burnstock 2001; Yamamoto, Shimizu et al. 2007; Chekeni, Elliott et al. 2010; Sridharan, Adderley et al. 2010; Orellana, Froger et al. 2011). ATP contained in granules could be released from platelets and during synaptic transmission (Falker, Lange et al. 2005; Gao, Wei et al. 2013; Shatarat, Dunn et al. 2014). ATP can also be released to the extracellular space by platelets (Beigi, Kobatake et al. 1999), lymphocytes (Schenk, Westendorf et al. 2008; Yip, Woehrle et al. 2009) or other cells (Bergfeld and Forrester 1992; Bodin and Burnstock 1996). Once in the extracellular media, ATP can elicit autocrine and/ or paracrine effects in monocytes and lymphocytes and act as a chemoattractant signal to monocytes and other cells (Piccini, Carta et al. 2008; Elliott, Chekeni et al. 2009; Yip, Woehrle et al. 2009; Manohar, Hirsh et al. 2012).

Extracellular ATP is rapidly degraded by the ectonucleotidase CD39 which degrades ATP to ADP and ADP to AMP (Deaglio and Robson 2011).

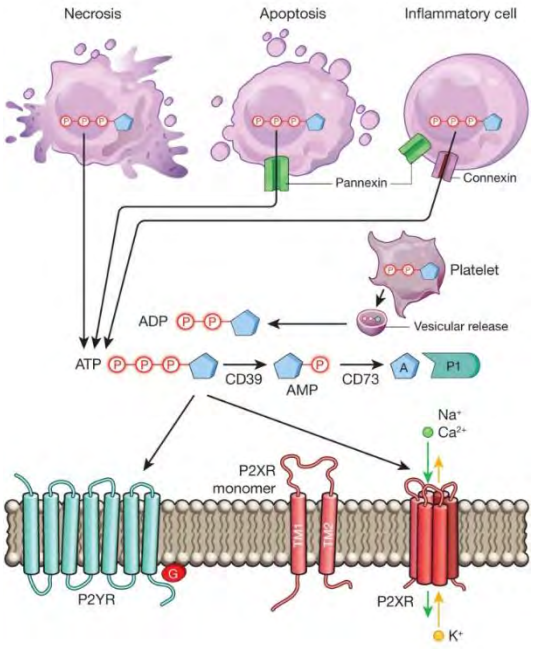

Figure 2. Extracellular nucleotide release and signaling during inflammation. Taken from: Idzko M. et al. Nature 509, 310-317 (2014).

Extracellular ATP is considered as an inflammatory signaling molecule that modulates various immune cells (Trautmann 2009). After tissue injury the purinergic signaling evolves in three phases. The first phase occurs during the first minutes or hours after the injury. In this phase damaged or stressed cells release ATP into the extracellular space where its concentration increases to high levels and starts the activation and attraction of immune cells and the inflammatory process. The second phase can last hours or days, is associated with a decrease in the ATP/adenosine ratio, the reduction of ATP mediated signaling and the increment in the activation of adenosine receptors A2A and A2B. The duration of

inflammation is determined by this phase. The third phase could last days or weeks, and is characterized by the beginning of the wound-healing process and low levels of adenosine and ATP. (Zawada, Rogacev et al. 2011; Heine, Ortiz et al. 2012; Cekic and Linden 2016).

The activation of P1 and P2 receptors elicit different effects on immune cells. It could result in anti-inflammatory responses, regulation of chemotaxis, release of IL-6 or mast cell degranulation (Hanley, Musset et al. 2004; Wu, Vadakkan et al. 2007; Bulanova and Bulfone-Paus 2010). P2 receptor activation responses will depend on the combination of P2 receptor expressed on each different immune cell. In lymphocytes ATP promotes p38 MAPK activation and increased IL-2 transcription resulting in cell proliferation and activation. If P2X7 receptor is locked by antagonist, it results in the transformation of naive CD4+T cells into T regulatory cells (Treg) after cell stimulation. ATP could also modulate the cytolytic activity of cytotoxic T cells. (Padeh, Cohen et al. 1991; Jacob, Perez Novo et al. 2013) Whereas the activation of P2X and P2Y receptors in monocytes results in the release of pro-inflammatory molecules, shedding of membrane antigens, activation of transcription factors related to inflammation, and chemotaxis (Ferrari, Wesselborg et al. 1997; Pizzirani, Ferrari et al. 2007; Wu, Vadakkan et al. 2007).

1.3 PURINERGIC SIGNALLING IN MONOCYTES AND MACROPHAGES

P2XRs and P2Yrs are expressed in all monocyte and macrophages cell lines. Their activation is important for the inflammatory process since it occurs during the

beginning of the inflammatory response and causes the activation and attraction of immune cells to the site of injury (Heine, Ortiz et al. 2012). P2X7 receptor activation and regulation has been related to the inflammatory process in monocytes and macrophages. In THP-1 monocytes its expression is modulated by pro-inflammatory molecules and by cAMP, a well identified anti-inflammatory mediator (Humphreys and Dubyak 1998). P2X7 activation by ATP in monocytes and macrophages has been related to the activation of a molecular complex named inflammasome which is implicated in the synthesis and maturation of IL-1β and IL-18 in cells previously exposed to LPS (Ferrari, Chiozzi et al. 1997; Mehta, Hart et al. 2001).

In macrophages, ATP permeates the plasmatic membrane to big fluorescent dyes, promoting cation fluxes (Steinberg, Newman et al. 1987), increasing the calcium currents (Sung, Young et al. 1985), inducing citotoxicity (Murgia, Pizzo et al. 1992) and cell lysis (Blanchard, McMillen et al. 1991). Also, P2X7 activation induces the death of macrophages infected with mycobacterium in a process mediated by the fusion of the phagosome with the lysosome elicited by calcium increase and PLD activation (Kusner and Adams 2000). P2X7 has also been involved in processes like Nitric Oxide Synthase expression and Nitric Oxide synthesis (Tonetti, Sturla et al. 1994), generation of Reactive Oxygen Species (Pfeiffer, Guerra et al. 2007) and leukotrienes (Ballerini, Ciccarelli et al. 2005). Besides P2X7 receptors, human alveolar macrophages express also P2X1, P2X4, P2X5, P2Y2, P2Y4, P2Y6, P2Y11, P2Y13 and P2Y14 receptors (Myrtek, Muller et al. 2008), but the

physiological response elicited by the activation of some of this receptors remains to be identified.

P2Y receptor activation mediate the transcription of IL-6 (Kaufmann, Musset et al. 2005), P2Y2 receptor activation induces the release of the chemokine CCL2, that recruits monocytes and other cells to the site of injury, and increases the expression of inflammatory genes (Wong, Yeap et al. 2012).

1.4 P2X RECEPTORS

P2X receptors are membrane ion channels permeable to cations that open in response to the binding of extracellular ATP. P2X subunits are encoded by seven genes (P2X1-P2X7) that form functional channels as trimeric structures (North 2002). All subunits are able to form functional channels as homomers with the exception of P2X6; also, some subunits could form heteromeric channels. Some reported heteromeric receptors include P2X2/P2X3, P2X4/ P2X6, P2X1/P2X5, P2X2/P2X6, P2X1/P2X4 and P2X4/P2X7, the last heteromeric receptor is very unstable and is unlikely to be expressed in cells (Saul, Hausmann et al. 2013). Each P2X subunit is formed by an intracellular amino terminal domain, two hydrophobic transmembrane regions (TM1 and TM2) an extracellular loop which contains the ATP binding site and one intracellular carboxy terminal region (Fig. 3). The subunits are conformed by among 384 aminoacids (P2X4) and 595 aminoacids (P2X7). The carboxyl terminal region is the most different among P2X subunits. Considering only the transmembrane regions and the extracellular loop, P2X subunits share 40 to 55% of homology (North 2002).

There are some conserved regions among P2X subunits. Ten cysteines are conserved in the extracellular loop of all subunits; it is believed that these cysteines become oxidized and thus contribute with the tertiary structure of P2X proteins by forming disulfide bridges. P2X subunits are glycosylated proteins. All subunits have glycosylation consensus sites (Asn-X-Ser/Thr), some of this glycosylations seems to be important for receptor transport to the cell membrane (Khakh 2001). Splicing variants of several subunits have been reported to be expressed in many tissues; the majority of these splicing variants have deleted exons or alternative acceptor/ donor sites (North 2002; Linan-Rico, Jaramillo-Polanco et al. 2012).

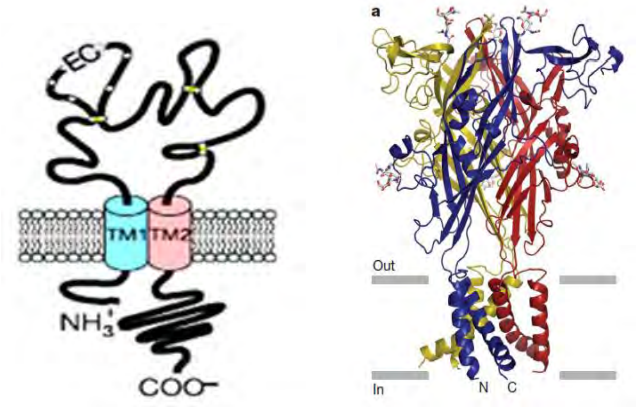

Figure 3. Cartoon representation of single P2X subunit and a P2X receptor assembled as a trimer. Each color represents a single subunit (right pannel). Taken from: Khakh B.S and North. Nature 442, 527-532 (2006) and Kawate et al. Nature 460, 592-599 (2009).

ATP induces fast activation of P2X receptors. The kinetics of the elicited response will depend on the P2X receptor characteristics. By using heterologous expression systems it has been possible the characterization of homomeric receptors. Homomeric P2X1 and P2X3 receptors showed fast desensitization when ATP was

applied. P2X2 and P2X4 showed a slow desensitization during the agonist exposure. Homomeric P2X7 receptor showed no desensitization in their responses, conversely, the current amplitude keeps increasing in the constant presence of ATP or after repeated exposure. A distinctive feature of P2X4 and P2X7 receptors is that both are able to form membrane pores after prolonged exposure to high ATP doses. P2X7 pore is associated with ionic flux across the membrane which could initiate the ATP associated apoptotic process. Another difference between P2X subunits is their sensitivity to ATP, P2X1 and P2X3 are highly sensitive to ATP since both receptors could be activated by ATP 1 μM. P2X2, P2X4 and P2X5 receptors could be activated with ATP 10 μM while P2X7 required concentrations higher than 100 μM to become activated. In native cells, the stimulation with ATP could reach a response with a unique kinetics due to the sum of responses mediated by the activation of all the P2X receptors expressed in each cell (North 2002).

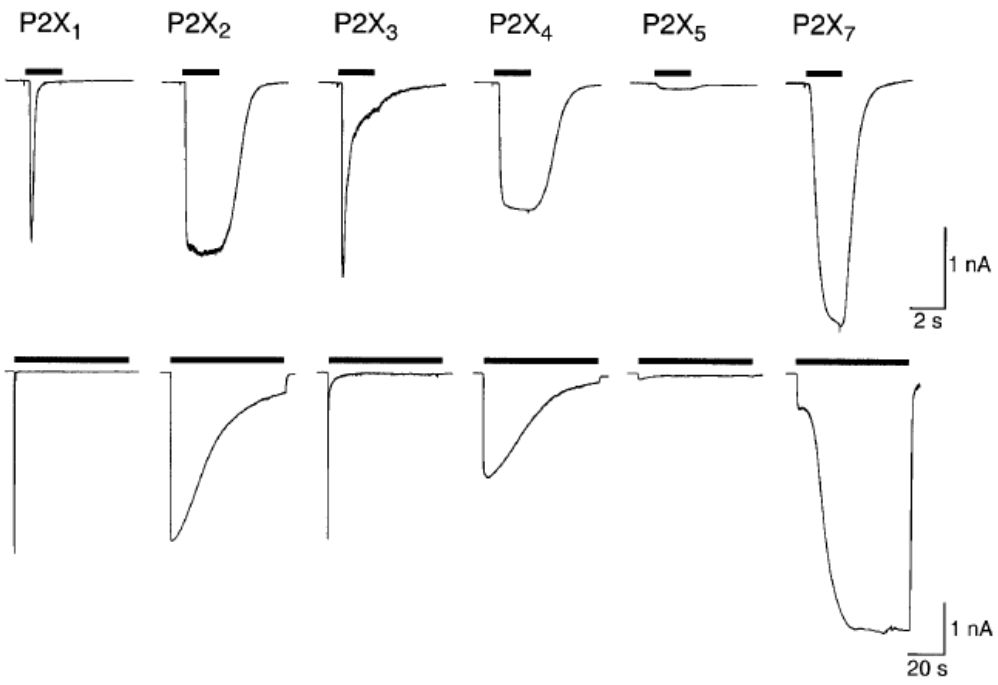

Figure 4. Fast and slow desensitization of homomeric rat receptors heterologously expressed in HEK cells. Taken from: North A. Physiol Rev. 82, 1013-1067 (2002)

2. BACKGROUND

Monocytes express P2X receptors and P2Y (G protein coupled) receptors. P2X receptors are ion channels permeable to cations located in the cell membrane that allow the ionic influx of Na+ and Ca2+ and the efflux of K+ following to the ATP binding to the receptor. This ionic flux induces plasma membrane depolarization, increment of intracellular Ca2+ levels with the subsequent activation of Ca2+ signaling cascades, like p38 MAPK or phospholipase A2 (Khakh and North 2006). Human monocytes express P2X1, P2X4 and P2X7 mRNA subunits (Wang, Jacobsen et al. 2004). However, there is no electrophysiological evidence of P2X activation in monocytes and an early study failed to record any P2X receptor mediated current in human blood leukocytes (Clifford, Parker et al. 1998). In macrophages, the P2X7 receptor activation has been implicated in the killing of mycobacteria, and pro-inflamatory events such as inflammasome activation and release of interleukin IL-1β, as well as in cell lysis and apoptosis (Ferrari, Chiozzi et al. 1997; Lammas, Stober et al. 1997; Ferrari, Los et al. 1999; Suzuki, Hide et al. 2004) but little is known about how the other subunits can be related to this processes.

In human macrophages, it has been shown that ATP activates membrane currents mediated by P2X receptors, responses that were attributed mainly to activation of P2X7 receptors, based on their pharmacological properties (Eschke, Wust et al. 2002). Nevertheless, when mouse peritoneal macrophages under whole cell configuration are activated with ATP, the elicited currents seems to be mediated by the activation of P2X1 and P2X4 homomeric receptor (Sim, Park et al. 2007;

Kessler, Clauss et al. 2011); P2X1 receptor is expressed in different blood cells; its activation has been associated with platelet aggregation, whereas the reduction of NF-kβ activation and TNF α release, and cell death in cells different to monocytes, (Chvatchko, Valera et al. 1996; Varani, De Mattei et al. 2010; Mahaut-Smith, Jones et al. 2011), have been related to P2X7 receptor activation (Ferrari, Wesselborg et al. 1997), in macrophages, P2X1 receptor is involved in HIV entrance to cells (Giroud, Marin et al. 2015).

It has also been reported that the monocytes P2X7 receptor expression increases during the differentiation process to macrophage *in vitro* (Gudipaty, Humphreys et al. 2001). These data suggest a regulation in the P2X subunit expression in blood monocytes and macrophages since the monocyte subpopulations appear to be different maturation stages during the monocyte to macrophage differentiation process (Gordon and Taylor 2005). P2X4 receptor has been associated with prostaglandin E release and pain and has been suggested that this receptor can also activate the inflammasome signaling (Ulmann, Hirbec et al. 2010).

The aim of this work was to investigate the distribution of functional P2X receptors and the isoform P2X1*del* in human monocytes. Our data show that in monocytes the expression of P2X subunits is heterogeneous and that currents elicited by ATP could be mediated by the activation of P2X1, P2X1*del*, P2X4 and P2X7. Furthermore, our data suggest that P2X receptor expression is upregulated during the monocyte to macrophage differentiation process. We also showed that 86% of the monocytes analyzed co-express P2X1 and a splicing variant named P2X1*del*,

which is able to form functional homomeric P2X1*del* channels. Currents mediated by these homomeric receptors increased in amplitude during the continuous presence of ATP, similar to those currents mediated by other P2X channels that are known to dilate and form membrane pores with high conductance.

3. THE PREMISE OF THIS STUDY

P2X receptors are involved in important physiological process related to the immune system. Their participation in the inflammatory response and pain has proposed them as possible therapeutic targets in the treatment of inflammatory diseases and as target for analgesics. Some studies have been conducted in order to characterize the native populations of P2X receptors in cells of the immune system, however it has not been completely elucidated how the P2X receptors expressed in human monocytes contribute to the elicited response after the stimulation with ATP.

In monocytes, the stimulation with ATP triggers a series of responses related to the activation of P2X7 receptor activation, recently it has been proposed that some of the effects could be mediated by the activation of P2X4 instead of P2X7. Eschke reported the characterization of ATP induced currents in human macrophages; they attributed the response obtained to the activation of P2X7 receptor. However, in mouse monocytes and macrophages the stimulation with different ATP concentrations elicited responses with fast or slow desensitization kinetics that are different to P2X7 and suggest the activation of P2X1 and P2X4 receptors.

Based on this information we studied the native composition of P2X subunits in human monocytes using electrophysiological and molecular techniques in order to correlate the currents elicited by ATP with the subunits expressed at the level of individual cells. This will help us to elucidate the native composition of P2X receptors in human monocytes and their participation in the ATP induced currents. An important part of the project is the characterization of P2X expression in

monocyte subpopulations and the search of splicing variants, which have been reported to be expressed in many human tissues.

4. HYPOTHESES

According to previously reported data, human monocytes express transcripts of P2X1, P2X4 and P2X7 receptors; however its transcription seems to be low.

5. GENERAL OBJECTIVE

To determine the presence of P2X1, P2X4 and P2X7 receptors and its splicing variants in single monocytes obtained from healthy subjects through flow cytometry and molecular and electrophysiological techniques.

6. SPECIFIC AIMS

- To study the expression of P2X1, P2X4 and P2X7 in human monocytes using Single Cell PCR, flow cytometry and whole cell patch clamp.

- To study the expression of the splicing variant P2X1*del* in human monocytes using Single cell PCR and heterologous expression.

- To study the possible formation of heteromeric receptors between P2X1 and P2X1*del* by heterologous expression.

7. MATERIALS AND METHODS

7.1 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from 4 ml of venous blood from healthy donors by a density gradient centrifugation. Blood was diluted 1:2 with phosphate-buffered saline (PBS) and layered over 2 ml of Ficoll-Hystopaque (Sigma, St Louis, MO) and centrifuged at 2500 rpm for 20 minutes. Cells were washed two times with PBS and suspended at $1x10^6$ cells/ml in RPMI-1640 medium (HyClone, Laboratories, Logan, UT), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). Monocytes were allowed to adhere in culture on sterile round coverslips at $5x10^5$ cell density at 37°C for at least 3 hours. Then, adherent monocytes were used for electrophysiological recordings or single cell extraction. All the experiments were done using monocytes cultivated for no longer than 24 hours after cell plating only in the presence of culture medium.

7.2 RT-PCR

Monocytes were purified using the anti-human CD14 antibody. Magnetic-activated cell sorter (MACS) separation columns (Miltenyi Biotec, Germany) protocol was used to obtain the monocyte population from PBMC according to the manufacturer's instructions. We collected $2x10^6$ monocytes in Eppendorf tubes with 1 ml lysis solution from RNAqueous RNA isolation kit (Ambion). Then, the RNA was obtained using an affinity column and collected for cDNA synthesis. We used 6 µg of mRNA and superscript reverse transcriptase II (Invitrogen) in the

presence of oligo (dT) for 1.5 h at 42°C. We performed PCR in 25 µl with 300-500 ng of cDNA, 0.2 µM dNTPs, 1-3.5 µM $MgCl_2$, one unit of Taq DNA polymerase (Invitrogen) and 0.2 µM oligonucleotides (see Table 1 for nucleotide sequence and Tm). We assessed the specificity of the designed nucleotides using the FastPCR software (Primer Digital Ltd.). Reaction conditions were as follows: Initial denaturation for 5 min at 94°C, then 35 amplification rounds composed of denaturation for 20s at 94°C, alignment for 20s at specific oligonucleotide temperature, extension for 1-1.5 min at 72°C; the final extension was 5 minutes at 72°C. We analyzed PCR products by electrophoresis in 1% agarose gels stained with 1 µg/ml ethidium bromide. Negative controls were performed with no cDNA template. Images were obtained with Gel-Doc 2000 documentation system (Bio-Rad).

7.3 Single cell PCR

Single monocytes were harvested under visual control into a glass pipette by applying negative pressure. Monocytes were selected and identified by their glass adherence properties (Eclipse TE200OU, Nikon). This pipette had a tip diameter of about 4-6 µm and contained 6 µl of RNase-free RTbuffer (with RNase inhibitor, 20U; oligo (dT)18, 2.3 mM; dNTPs, 150 mM; dTT 1.2 mM; 10X RT Buffer Superscript III First-Strand Synthesis System; Life Technologies, Texas,USA). The content of the pipette was expelled into a PCR-tube containing 12 µl of RNase-free RT buffer and 0.5 µl of NP40 1% to allow cell membrane solubilization and the reaction was incubated at 65°C for 2 min. After adding 0.5 ml of reverse transcriptase III, the sample was transferred to 37°C for 60 min, the reaction was

inactivated by heating the sample to 70°C for 10 min and placed on ice. Negative controls were performed without template; no false amplifications were obtained. Amplification of the human P2X1 receptors was performed by nested PCR, using the following primers and conditions; for the first P2X1 PCR we used the primers pair X1-F1 + X1-R1 obtaining a 1.33 kb PCR product (table 1). For nested PCR 1 µl of the first PCR was used as template and using primers X1-F2 + X1-R2 we obtained a 627 bp and a 576 bp fragments. For the specific amplification of the splicing variant we performed another nested PCR using 1 µl of the first reaction and primers X1-F3 + X1-R2 obtaining a PCR product of 197 bp (table 1). Primer pairs used in the first and nested PCR were located at the UTRs region and inside the ORF, respectively. Both PCR were performed in a 2720 thermal cycler (Applied Biosystems) using Taq DNA Polymerase (Life Technologies, Texas, USA). The PCR conditions were: 1 cycle at 94°C (5 min), then 35 cycles of 94°C (15 s), 60°C (or 56°C for nested PCR, or 60 °C for splicing variant, 15 s), 72°C (1min 45 s for first PCR, and 45 s for nested PCR), followed by 72°C (5 min) for final extension. Amplification of P2X7 receptor was performed using the oligonucleotides X7-F2 + X7-R2 for the first amplification round obtaining a PCR product of 1165 bp. Nested PCR was performed using oligonucleotides X7- F3 and X7-R2 obtaining a PCR product of 886 bp by using similar PCR conditions with the adequate alignment temperature for each oligonucleotide pair. Amplification of P2X4 receptor was performed using the oligonucleotides X4-F1 + X4-R1 for the first amplification round obtaining a PCR product of 1336 bp. Nested PCR was performed using oligonucleotides X4-F2 and X4-R2 obtaining a PCR product of 872 bp, Using 1 µL of a 1:10 of the first nested PCR as template, a second nested PCR was made

using the oligonucleotides X4-F3 + X4-R3 obtaining a PCR product of 543 bp by using similar PCR conditions with the adequate alignment temperature for each oligonucleotide pair. The amplification products were analyzed by ethidium bromide staining subsequent to agarose gel electrophoresis (1.5%). To verify the identity of amplified mRNA, these PCR products were sequenced by MCLAB DNA sequencing service (San Francisco City, CA). The size of each of the PCR amplicons was predicted according to its corresponding cDNA sequence submitted at the NCBI GenBank.

7.4 Flow cytometry analysis of P2X1, P2X4 and P2X7 receptors in human monocytes

In all experiments, we used freshly isolated PBMC ($5x10^5$), which were fixed into 1% PFA and permeabilized with 0.1% saponine (SIGMA) for 5 min and monocytes were gated based on size and granularity (Forward Scatter (FSC) and SideScatter (SSC) properties) typical of these cells and the quadrants of the dot plot were adjusted according to not staining cells in control experiments (see Fig. 7A). In the rest of experiments, PBMC were incubated with mouse antihuman CD14 FITC labeled antibody and mouse antihuman CD16 PE antibody for 20 min (BD Pharmigen) in the dark and also fixed and permeabilized as described above (see Fig. 7B). Different sets of cells were also incubated with rabbit antihuman P2X1 polyclonal antibody (Alomone labs, Israel) directed to the receptor c-terminus, corresponding to amino acids 382-399 of rat P2X1, or rabbit anti human P2X4 polyclonal antibody (Santa Cruz Biotechnology, or Biorbyt) directed to the receptor N-terminus or rabbit anti human P2X7 polyclonal antibody (Calbiochem,

Billerica,MA) directed to the receptor c-terminus, every antibody was added for 30 minutes at 4°C in the dark. The cells were washed and then incubated with a secondary goat anti-rabbit APC antibody (Santa Cruz Biotechnology, Dallas Texas) for 20 min at 4°C in the dark. Cells were fixed with 1% PFA and stored at 4°C until analysis by flow cytometry on a FACS Canto flow cytometer using the Cell Quest software (Becton Dickinson, San Jose, CA). To investigate specificity of the primary anti-P2X1, anti-P2X4 and anti-P2X7 antibodies, we also carried out experiments without those antibodies (see Fig. 7C). For the analysis of P2X receptors in monocyte sub-populations, cells were identified by their FSC and SSC properties and P2X receptors expression was evaluated in the selected sub-populations defined by their expression of CD14 and CD16.

7.5 Patch-clamp recordings

Whole-cell patch-clamp recordings were made with the Axopatch 200B amplifier (Molecular Devices, Sunnyvale,CA, USA). Recordings were carried out at room temperature (22-24°C). Patch pipettes were made as previously described (Barajas-Lopez, Huizinga et al. 1996) and their resistance was between 5 and 7 MΩ. During current recordings the membrane potential was held at −40 mV. Currents were stored in a hard drive of a PC using AXOSCOPE 9 (Molecular Devices, Sunnyvale, CA, USA) at a sampling frequency of 1 kHz and analyzed using AXOGRAPH 4.9 software (Molecular Devices, Sunnyvale, CA, USA). The standard external solution contained (in mM): NaCl, 160; CaCl2, 1; glucose, 11; HEPES, 4.8 and CsCl, 3; the pH was adjusted to 7.3–7.4 with NaOH 10N. ATP stock solutions (100 mM) were prepared in water before the experiments and the

pH was readjusted with NaOH to 7.3. The standard pipette solution consisted in (mM): CsCl, 140; EGTA, 10; HEPES, 5; NaCl, 10; ATPMg, 4.5 and GTPNa, 0.1; adjusted to pH 7.3–7.4 with CsOH. Coverslips with monocytes were fix into a recording chamber and continuously superfused with standard external solution at about 2 ml/min. Rapid application of ATP was achieved using an eight-tube device (tubes used were 0.5 mm of external diameter). Each of these tubes was connected to a syringe containing the control or an experimental solution. The control solution tube was placed ~300 μm in front of the cell being recorded and ATP application was done by abruptly moving another tube in front of the cell, which was already draining the same control solution plus ATP. Tubes were moved using a Water Robot Micromanipulator (WR-88; Narishigue Scientific Instrument Lab, Tokyo Japan). ATP was removed by returning to the control solution tube. External solution was released by gravity and the level of the syringes was frequently adjusted to minimize changes in flow rate (about 0.5 mL/min).

7.6 Cloning of P2X1 receptor

PCR was performed using human P2X1 primers designed at the 5' and 3' UTRs regions to amplify the entire coding sequence including the Kozak sequence, from the first single cell PCR reaction as previously described. The primers used were: forward (F) 5'-**GGATCC**AGCCGGCCCACCATGG-3'; and reverse (R) 5'-**GAATTC**ACGCTGCACCCAGTCAGGAGTT-3'. PCR reaction was done using Platinum Pfx Taq DNA Polymerase (Life Technologies, Texas, USA), conditions were as follows: initial denaturation for 5 min at 94 °C, then 40 amplification rounds of denaturation for 15 s at 94°C, alignment for 20 s at 60°C, and extension for 1

min 45 s at 68°C; the final extension was 5 min at 68°C. PCR products were cloned into the pGEM-T Easy Vector (Promega, Wisconsin, USA) sequenced and subcloned in pCDNA3 vector. The nucleotide sequences were confirmed by a minimal of three sequencing rounds using different primers.

7.7 Xenopus leavis oocyte experiments

Frogs were anesthetized by immersions in a solution of 10 mM Tricaine (3-aminobenzoic acid ethyl ester; Sigma-Aldrich, MX) and oocytes were removed by dissection. Stages V-VI oocytes were manually defolliculated and placed in Barth's solution (NaCl, 88 mM; $Ca(N0_3)_2$, 0.33 mM; KCl, 1 mM; $CaCl_2$, 0.41 mM; $MgSO_4$, 0.82 mM; $NaHCO_3$, 2.4 mM; and HEPES, 10 mM pH adjusted to 7.2-7.4 with NaOH). Cells were injected with 36 nL (15 ng) of cap and poliA mRNA (P2X1 and P2X1*del*, alone or together 1:1 ratio) and incubated at 14°C for 12-36 h before the electrophysiological experiments. The cap and poliA mRNAs were synthesized with T7 mMessage mMachine (Life Technologies, Texas, USA). The mRNAs were dissolved in RNase- free water at a final concentration of approximately 400 ng/µl, aliquoted, and stored at -70°C until used.

Membrane currents of oocytes were recorded using the two-electrode voltage clamp and the Axoclamp 2B amplifier (Molecular Devices) at a sampling frequency of 1 kHz. As in patch clamp experiments, currents were stored in a hard drive of a PC using AXOSCOPE 9 (Molecular Devices, Sunnyvale, CA, USA) and analyzed using AXOGRAPH 4.9 software (Molecular devices, Sunnyvale, CA, USA). Recording electrodes consisted in glass pipettes (0.3-0.8 MΩ resistance) filled with

2 M KCl solution containing 10 mM EGTA. ATP-induced currents (I_{ATP}) were recorded at a holding potential of -60 mV and at room temperature (21-25°C). For most experiments, at least otherwise stated, application of ATP was done for ~10 sec or until the current reach a peak, and then washed off for 5 min. ATP solutions were freshly prepared and maintained in ice to decreased degradation. The recording chamber was continuously superfused with standard external solution (NaCl, 88 mM; KCl, 2 mM; CaCl2, 1 mM; MgCl2, 1 mM; and HEPES, 5 mM pH adjusted to 7.2-7.4 with NaOH) at approximately 3 ml/min. Drug application around the recorded cell was achieved by rapidly exchanging the external solution with one containing the drug by using an eight-tube device (tubes have an external diameter of 1.6 mm). Tubes were placed ~1 mm in front of the oocyte being recorded and ATP application was done by abruptly moving another tube in front of the cell, which was already draining the same control solution plus ATP (flow rate 1.5 mL/min). Tubes were moved manually using a Narishigue manipulator (MMN-3).

7.8 Data analysis

In each oocyte, we normalized I_{ATP} considering the response to ATP 100 μM as 100% for P2X1 and the response to ATP 5 mM for P2X1*del*, whereas, in monocytes the response to ATP 30 μM was considered as 100%. Data are expressed as the mean ± the standard error of the mean (S.E.M.), the number of cells used is represented by *n* and concentration–response curves were fitted with a three parameters logistic funtion (Kenakin 1993) using KaleidaGraph 4.1.0 (Synergy Software).

8. RESULTS

8.1 P2X1 mRNA is expressed in human monocytes.

We performed RT-PCR from purified blood monocytes and lymphocytes cDNA with specific oligonucleotides to amplify the complete open reading frame (ORF) of the P2X1 receptor (Fig. 5a). Using oligonucleotides X1-F1 and X1-R1 (Table 1) we obtained a PCR product of 1.33 Kb (Fig. 5b). Receptor characteristics were confirmed by sequencing and 100% of identity was found with the NCBI reported sequence (Access Number NM_002558.3). Besides the canonical P2X1 sequence, we obtained from monocytes cDNA a P2X1 sequence lacking 51 bp corresponding to the first part of the exon 6. The sequence corresponded to a splicing variant previously reported (Greco, Tonon et al. 2001), expressed in human platelets and named P2X1*del* (Fig. 5a). To determine if this splicing variant is co-expressed with the canonical receptor in monocytes, we performed single cell RT-PCR. Using nested PCR and oligonucleotides X1-F2 + X1-R2 (Table 1) we were able to amplify both, the P2X1 mRNA in 90% of the monocytes analyzed (n=54 from 7 healthy subjects), whereas, P2X1*del* was found in 88% of the monocytes (n= 36 cells from 4 healthy subjects, Fig. 5c, 5d), the later average was obtained from cells in which the presence of P2X1*del* was confirmed using the X1-F3 oligonucleotide (Table 1, Fig. 5a) designed to match the last 12 nucleotides of exon 5 and 6 nucleotides of exon 6 (ORF nucleotides 577-582 in P2X1 sequence: NM_002558.3). Identity of the 197 bp PCR product was confirmed by sequencing (Fig. 5a-c). In the same 36 monocytes tested for both mRNAs we found P2X1 in 34 and co-expressed P2X1*del* in 31 cells (86%, Fig. 5d). P2X1 receptor transcripts were found in monocytes from all subjects tested.

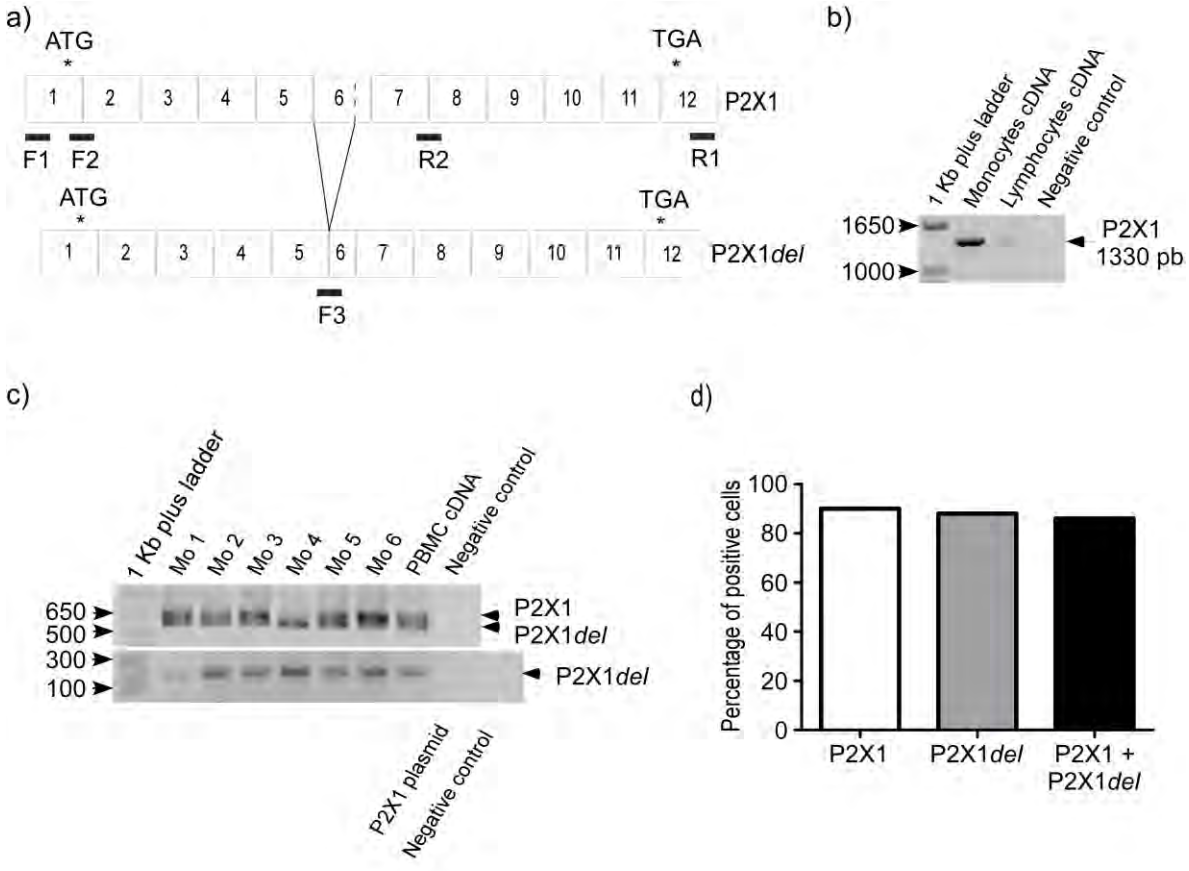

Figure 5. Most human monocytes express P2X1 and P2X1*del* mRNA. Using RT-PCR and Single Cell PCR, we amplified the complete ORF of P2X1 subunit and a splicing variant P2X1, which lacks 51 bp at the exon 6 in monocytes (P2X1*del*). **a)** Schematic representation of the exon-organization of P2X1 and P2X1*del* mRNAs and primer locations (X1-F1, X1-F2, X1-R1, X1-R2 and X1-R3); note that some primers were designed between different exons to avoid the amplification of genomic DNA. **b)** Agarose gel electrophoresis (1,5%) of P2X1 RT-PCR amplification from monocytes and lymphocytes; negative control was performed without template. **c)** Single cell RT-PCR performed with three sets of primers that distinguish P2X1 (X1-F2 + X1-R2) or P2X1*del* (X1-F3 + X1-R2). We used cDNA from Peripheral Blood Mononuclear Cells (PBMC) as positive control and negative control was done without template. To confirm the specific amplification of the splicing variant using the primers stated, we performed a PCR using the complete ORF of P2X1 receptor cloned in pGEM-T Easy, no amplification was obtained **d)** Percentage of monocytes expressing each P2X1 subunit and co-expressing both subunits.

8.2 Heterogeneous expression of P2X mRNA receptors in human monocytes.

We performed RT-PCR from purified blood monocytes cDNA with specific oligonucleotides to amplify P2X4 and P2X7 receptors mRNA. Using oligonucleotides X7-F1 + X7-R1 (table 1) we obtained a PCR product of 1.1 Kb and with oligonucleotides X4-F2 + X4-R2 (table 1), we obtained a PCR product of 0.87 Kb corresponding to P2X7 and P2X4 receptors respectively (Fig 6a). Receptors characteristics were confirmed by sequencing and 100 % of identity was found with the NCBI reported sequences (P2X4 Access Number: NM_002560.2; P2X7 Access Number: NM_002562.5). In order to search the distribution of P2X4 and P2X7 on individual monocytes, we performed single cell PCR with the monocytes cDNA previously used to describe the expression of P2X1 and P2X1*del* receptors, so, we assessed the expression for the three P2X receptors and the P2X1 splicing variant on the same cells. Using nested PCR and oligonucleotides X4-F3 + X4-R3 for P2X4 and X7-F3 + X7-R2 for P2X7 (Table 1). We were able to amplify P2X4 mRNA in 85% of the monocytes analyzed, whereas, P2X7 was found only in 3 % of the monocytes (n=54 from 7 healthy subjects, Fig. 6 b-c). Two nested rounds were needed to amplify the P2X4 receptor. Identity of the 543 bp (P2X4) and 886 bp (P2X7) PCR products was confirmed by sequencing. This individual monocyte analysis showed that there is a heterogeneous expression of the P2X receptors in monocytes (Fig 6b, 6d). Our data shows that 76% of the monocytes co-express mRNA of P2X1 and P2X4, 9% express only P2X4 and 15% express only P2X1 (Fig. 6d). We found just one event where the mRNA for P2X1, P2X4 and P2X7 receptors were co-expressed. P2X1*del* was co-expressed with P2X1 and P2X4 in 70% of the cells tested (n= 35 from 4 healthy subjects, Fig. 6b).

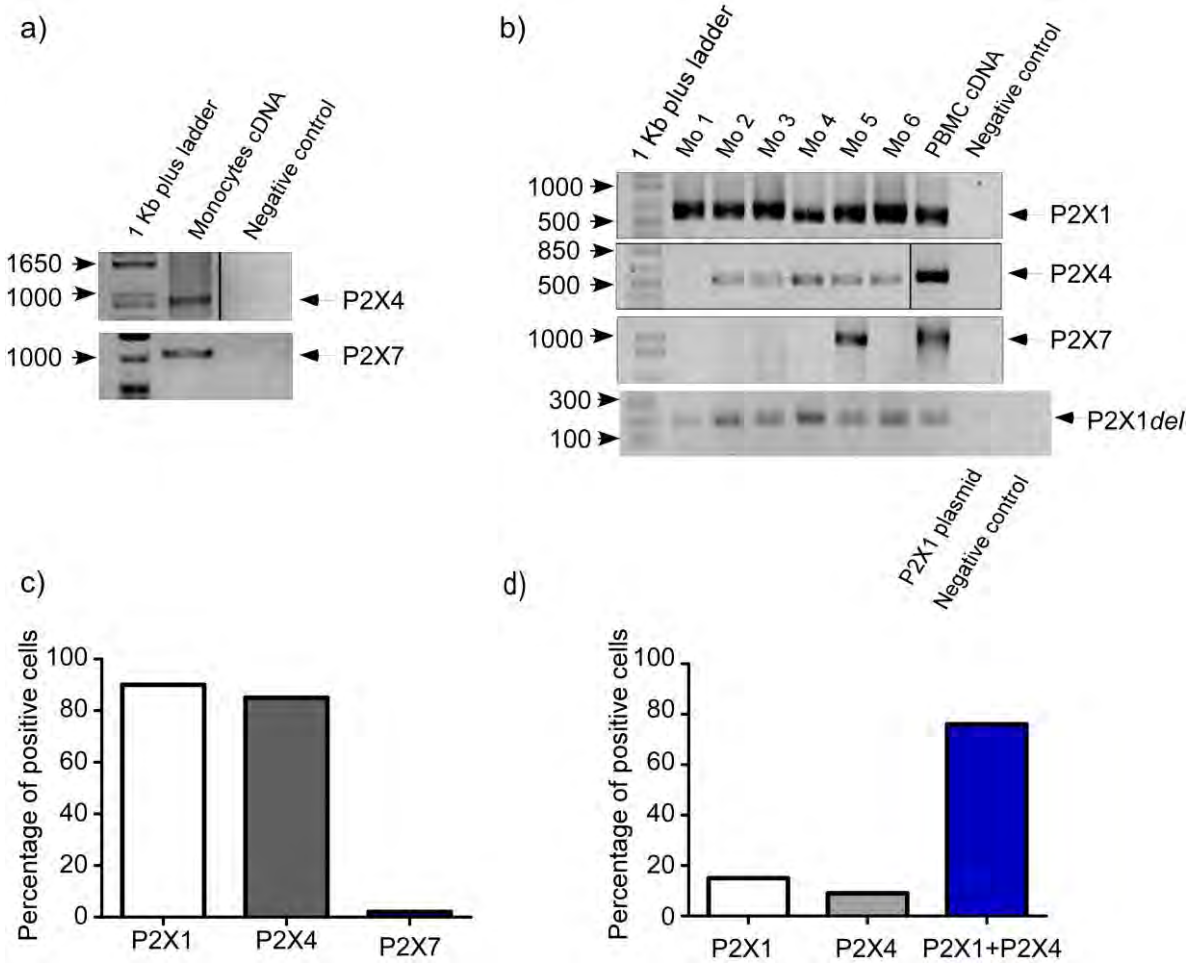

Figure 6. **Heterogeneous expression of P2X subunits in single monocytes.** Using RT-PCR and single cell PCR, we amplified fragments of P2X4 and P2X7 receptors mRNA in monocytes expressing P2X1 and P2X1*del* receptors. **a)** Agarose gel electrophoresis (1.5%) of P2X4 and P2X7 RT-PCR amplification from monocytes; negative control was performed without template. **b)** Single cell RT-PCR performed with four sets of primers that distinguish P2X1 (X1-F2 + X1-R2), P2X4 (X4-F3 + X4-R3), P2X7 (X7-F3 + X7-R2) or P2X1*del* (X1-F3 + X1-R2). We used cDNA from Peripheral Blood Mononuclear Cells (PBMC) as positive control and negative control was done without template. **c)** Percentage of monocytes expressing P2X1, P2X4 and P2X7 receptors. **d)** Percentage of monocytes co-expressing P2X1 and P2X4 receptors, or expressing only P2X1 or P2X4 receptors.

8.3 P2X1 protein is expressed in human monocytes.

We evaluated the expression of P2X1 receptor in monocytes from healthy subjects by flow cytometry. Monocytes were stained with an antibody directed to CD14 and identified by their forward and side scatter characteristics. In the gated population of monocytes we evaluated the expression of P2X1 with an antibody directed to the C-terminus portion of the receptor and developed with an APC secondary antibody (Fig. 7a-d). We found that 70±4 % (11 different subjects) of $CD14^{+}$ monocytes, expressed P2X1 receptor protein (Fig. 7e-f). The lost region of P2X1*del* is extracellular and, therefore,most likely this antibody does not distinguish between the two isoforms. The percent of monocytes expressing P2X1 protein is lower than those expressing the P2X1 receptor mRNA, which could be the result of higher false negatives observations by flow cytometry or due to the fact that mRNA levels do not predict perfectly the levels of protein expression in monocytes, as it has been previously shown (Guo, Xiao et al. 2008).

8.4 P2X4 and P2X7 proteins have lower expression than P2X1 in human monocytes.

Following with the analysis of P2X expression we determined by flow cytometry the expression of P2X4 and P2X7 receptors in monocytes CD14+ from the same healthy donor samples used to determine the P2X1 protein expression. Monocytes were identified as previously described and the expression of P2X4 and P2X7 evaluated with the antibodies as stated in materials and methods. We found a lower number of monocytes expressing P2X4 and P2X7 receptors than P2X1, only the 13±1.4% of the monocytes expressed P2X4 whereas P2X7 receptor was

detected only in 8±1% of the monocytes from 13 different healthy subjects (Fig 7f). The percent of monocytes expressing P2X4 protein is lower than those expressing the P2X4 receptor mRNA, this result was obtained with both antibodies directed to P2X4 tested, suggesting there is a lower level of translation of P2X4 than of transcription. The percent of monocytes expressing P2X7 protein is as low as those expressing the P2X7 receptor mRNA.

8.5 P2X1 and P2X7 proteins are expressed in a larger number of Non Classic monocytes (CD14+CD16+).

We also determined the P2X receptor distribution between monocyte subpopulations. It has been proposed that the monocyte subpopulations represent different maturation stages in the process of monocyte to macrophage differentiation. Furthermore, it has been reported that P2X7 receptor expression increases in the monocyte to macrophage differentiation process in vitro (Ziegler-Heitbrock L. 2007, Gordon and Taylor 2005, (Gudipaty, Humphreys et al. 2001). In order to detect changes on P2X receptors expression in monocyte subpopulations, we evaluated the expression of P2X1, P2X4 and P2X7 in monocytes stained with antibodies for the detection of CD14 and CD16 to identify and differentiate the Classic monocytes (CD14+CD16-) versus the Non-Classic monocytes (CD14+CD16+) (Fig. 8a). We found that the non classical monocytes (CD14+CD16+) express the P2X1 (81±3.7% vs 68.5±3.6 %, n=11) and P2X7 receptors (10.8±1.6 vs 6.9±0.9 %, n=13) in a higher percentage of cells compared to classic monocytes (CD14+CD16-). P2X4 receptor expression was not different among subpopulations (15.9±1.2 vs 12.6±1.5 %, n=13) (Fig. 8b-c). The mean

fluorescence intensity (MFI) values for each P2X receptor expression were not different among monocyte subpopulations (Fig. 8d), these data suggest that P2X expression is modulated during the differentiation process from monocyte to macrophage but not the expression level of the receptors.

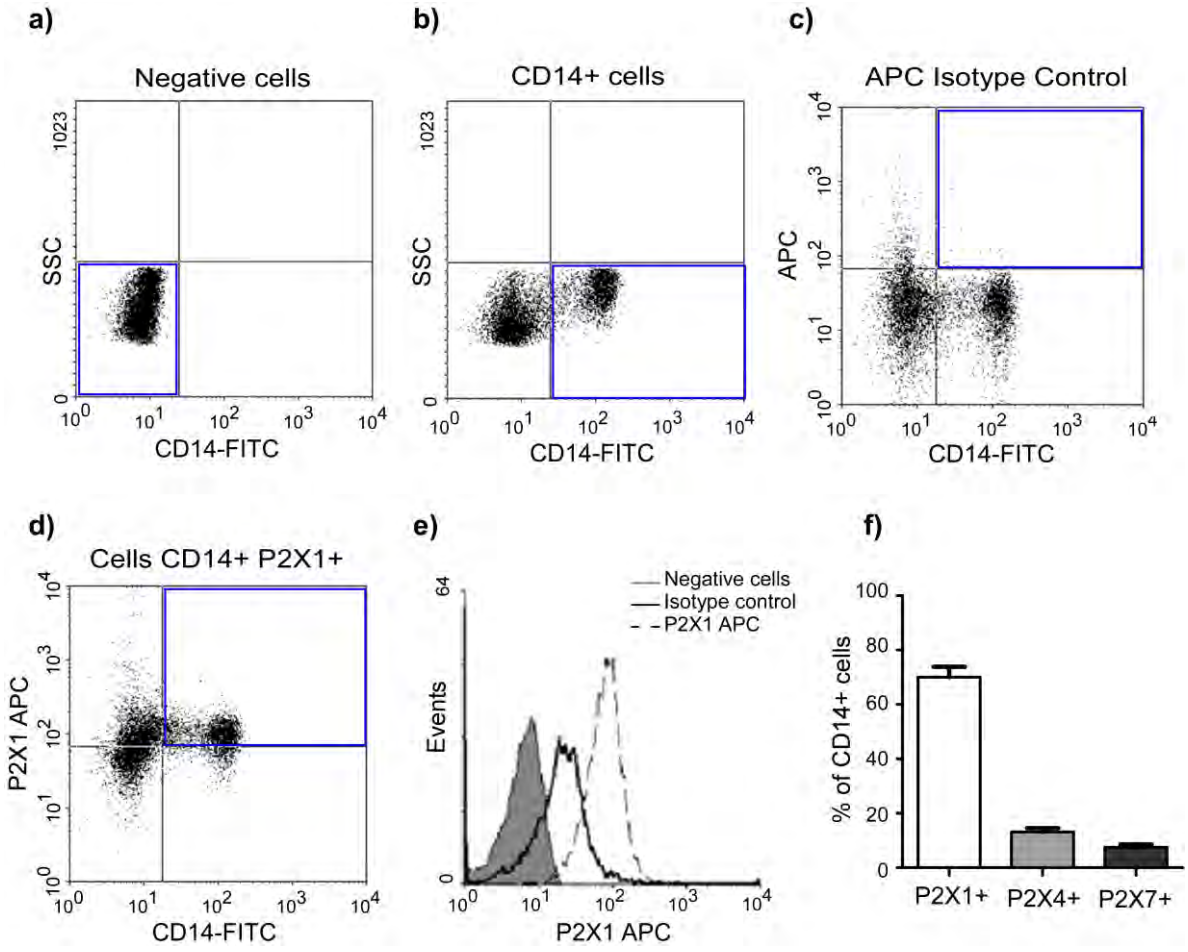

Figure 7. P2X1 receptor protein is expressed in most human monocytes. Peripheral Blood Mononuclear cells (PBMC) from healthy subjects were immunostained for the detection of P2X1 receptor in CD14+ monocytes and analyzed by flow cytometry. Monocytes were identified by their Forward Scatter (FSC) and Side Scatter (SSC) properties and CD14 staining (see methods for details). **a)** Dot plots of cells incubated without anti-CD14-FITC antibody (control). **b)** Dot plots of

monocytes incubated with anti-CD14-FITC. CD14+ cells are indicated by the lower right (blue) rectangke. PBMC, incubated for anti-Cd14 antibody, were either exposed to the secondary antibody (APC; **c)**, or incubated with anti-P2X1 antibody, **d)** as stated in methods). Cells co-expressing CD14+ P2X1 preoteins are indicated by the upper right (blue) rectangle. **e)** Histogram of P2X1+ cells in the monocyte gate. **f)** Percentage of cells expressing P2X1, P2X4 and P2X7 receptors in CD14+ cells from 11-13 healthy subjects.

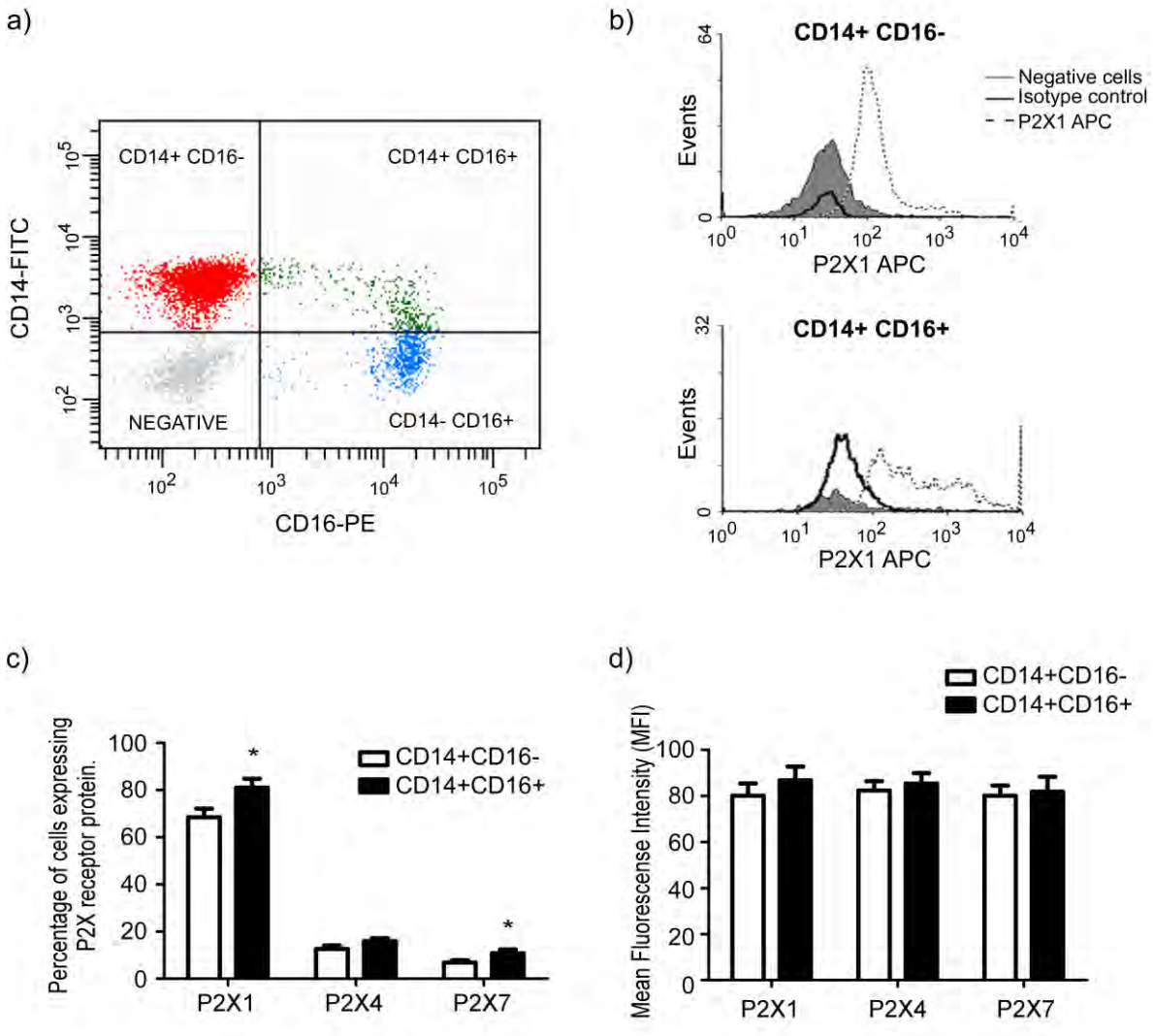

Figure 8. ***Expression of P2X1, P2X4 and P2X7 receptors in human monocytes.*** Peripheral Blood Mononuclear cells (PBMC) from healthy subjects were immunostained for the detection of P2X1, P2X4 and P2X7 receptors and analyzed by flow cytometry, Monocytes were identified by their Forward Scatter (FSC) and Side Scatter (SSC) properties and CD14/ CD16 staining to identify subpopulations as stated in materials and methods. **a)** Representative dot-plot of monocyte subpopulations; Classic (CD14+CD16-) and Non-Classic (CD14+CD16+). **b)** Histogram of P2X1+ cells in CD14+CD16- and CD14+CD16+ monocytes. **c)** The Non-Classical monocytes (CD14+CD16+) express P2X1 and P2X7 receptors in a higher percentage of cells. **d)** The mean fluorescence intensity of P2X1, P2X4 and P2X7 receptors is not different between classic and non-classic monocytes (n=11-13 healthy subjects).

8.6 Heterologous expression of P2X1 or P2X1del mRNAs

Expression of P2X1 or P2X1*del* mRNAs in *Xenopus leavis* oocytes formed functional homomeric channels. P2X1 receptor showed the previously reported characteristics of P2X1 homomeric receptor: high ATP sensitivity (EC_{50}=1.9±0.8 μM), activation at low ATP concentrations (0.1 μM), a maximal concentration of 10-30 μM ATP (Fig. 9a), fast desensitization kinetics (Tau 0.65±0.12 s, n=4, Fig. 9b and 9d), and desensitization > 90% (Fig. 10d). At higher ATP concentrations (3-5 mM), the current amplitude of the canonical receptor continued increasing after the fast desensitization component (Fig. 9d). P2X1*del* receptor was unresponsive to low ATP concentrations (≤100 μM, Fig. 9a) but when higher ATP concentrations were applied, we recorded small inward currents with quite different kinetics than the reported for the P2X1 canonical receptor (Fig. 9c). We did not see any fast transient current. Indeed, P2X1*del* responses did not show desensitization and when exposed to 3 and 5 mM ATP, the current amplitude increased under the continuous stimulation (Fig. 9c), similarly to what happens with the canonical P2X1 receptor at these high concentrations (Fig 9d). However, not inward current was observed, even by high ATP concentrations (3-5 mM), in 8 oocytes injected with control solution without mRNA (Fig 9c).

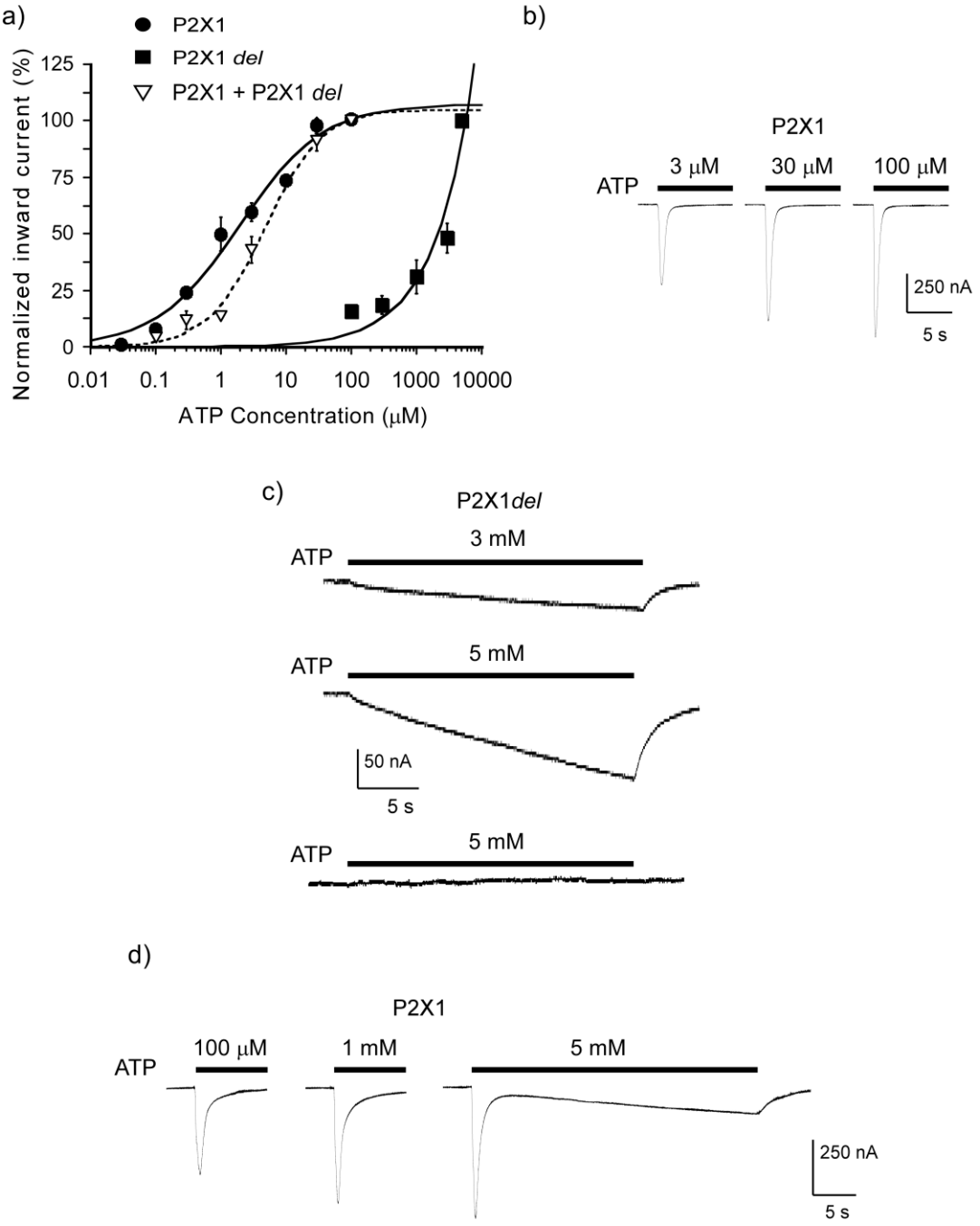

Figure 9. Properties of currents mediated by P2X1 and P2X1*del* channels heterologous expressed in *Xenopus leavis* oocytes. a) Concentration-response curves for receptors: P2X1 (EC_{50}=1.9±0.8 μM), P2X1*del* (EC_{50} >1000 μM) and oocytes co-expressing P2X1 and P2X1*del* (EC50 4.65 ± 1.34 μM). Each symbol represents the average value of 3 to 12 experiments and the associated lines are S.E.M. **b)** P2X1 channels are highly sensitive to ATP and their inward currents

rapidly desensitized. **c)** Currents mediated by P2X1*del* receptors are activated only at high ATP concentrations and show not desensitization; indeed, current amplitudes keep increasing during the continuous presence of ATP. No response was observed in oocytes injected with control solution without mRNA. **d)** Inward currents induced by high ATP concentrations in oocytes expressing P2X1 receptors, current amplitudes keep increasing during the continuous presence of ATP. Oocytes were clamped at -60 mV.

8.7 Co-expression of P2X1 and P2X1del mRNAs

When P2X1 and P2X1*del* receptors were co-expressed in the same oocyte and low ATP concentrations were used, ATP evoked an inward current with fast desensitization kinetics but with different properties than the observed for P2X1 canonical homomeric channels (Fig. 10a). Oocytes co-expressing both receptors showed lower ATP sensitivity (EC_{50} 4.65 ± 1.34 μM) than the oocytes expressing only P2X1 (Fig 9a). When higher ATP concentrations were tested, we also observed the increasing current after the fast desensitization component (Fig. 10b), but in this case, the increase in the response (100 μM: 5mM) was bigger in the cells co-expressing the receptors compared to cells expressing only the canonical receptor (P2X1, 100:140 %, versus P2X1 + P2X1*del*, 100:200 %, Fig 10f). Oocytes co-expressing both receptors showed different desensitization properties than the canonical receptor, suggesting the formation of heteromeric receptors (Tau 1.71 ±0.26 s, n=10, lower percent of desensitization, Fig. 10d and 10e). The oocytes co-expressing P2X1*wt* + P2X1*del* have smaller and more stable currents (343.8 ± 34.12 nA, n=13) than the cells expressing only the wild type receptor (895 ± 173.1 nA, n=12, Fig 10f).

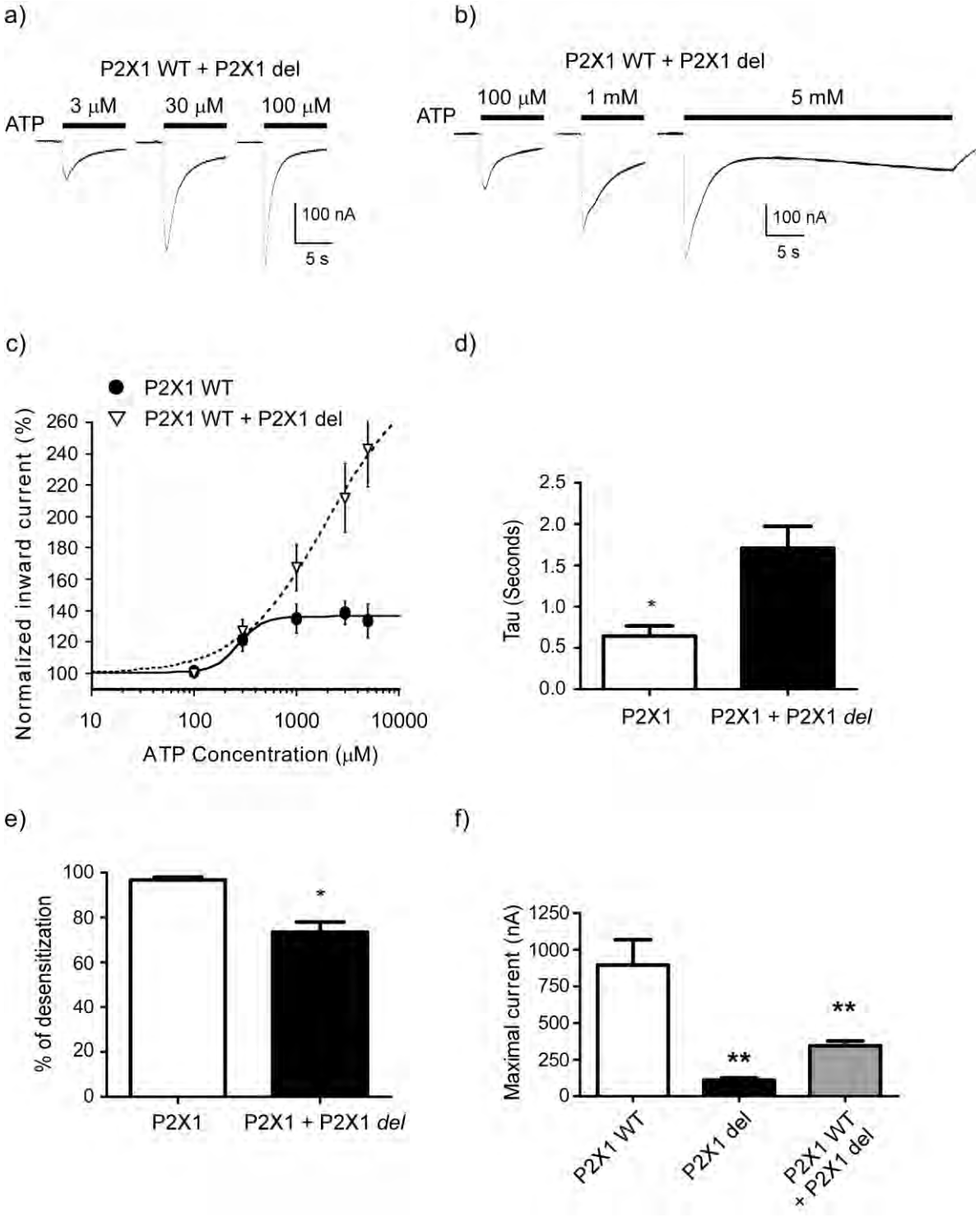

Figure 10. Properties of currents mediated by P2X1 and P2X1*del* channels co-expressed in *Xenopus leavis* oocytes. a) Currents elicited by low ATP application on oocytes co-expressing P2X and P2X1*del* showed different kinetics than the ones expressing only P2X1. **b)** Recordings obtained when higher ATP concentrations were applied, current amplitudes keep increasing during

the continuous presence of ATP. **c)** The current increment in continued presence of ATP is bigger in oocytes co-expressing P2X1 and P2X1*del.* ***d*****)** Co-expression of P2X1 receptors evokes inward currents with bigger Tau and lower desensitization than P2X1 **(e)**. **f)** In oocytes co-expressing P2x1 receptors the current amplitude elicited by ATP has lower amplitude but higher stability than oocytes expressing P2X1. Oocytes were clamped at -60 mV.

8.8 Evidence of P2X1 receptor participation in ATP induced currents in monocytes

To investigate if functional P2X1 receptors are expressed in human monocytes we used the whole-cell configuration of the patch clamp techniques. ATP was applied for 5-10 seconds at 5 min intervals unless otherwise indicated. Monocytes were voltage clamped at -40 mV and ATP application induced an inward current at concentrations ≥3 μM. At ATP concentrations ≤500 μM, we recorded a rapidly desensitizing current (upper left panel of Fig 11b), which was well fitted with a single exponential function (Tau 0.75±0.29 s, n=9) in cells that were stimulated with either 30 or 100 μM. ATP concentration-response curve for such a current is showed in Fig 11a, with an EC_{50} of 6.3±0.2 μM, this value was significantly larger ($P<0.001$, unpaired student´s t tes, two tails) than that observed in oocytes expressing the P2X1 homomeric receptors (EC_{50}=1.9±0.8 μM). In some cells, we observed currents with lower desensitization and a different Tau (>1s) (Fig 11b. upper right panel) suggesting the expression of P2X4 receptor. At high ATP concentrations (≥ 1 mM), current amplitude kept increasing during the agonist exposure, sometimes after a fast desensitizing current (Fig. 11b, lower left panel) or right after the current onset (Fig. 11b, lower right panel), similar to what it was

observed for both P2X1 isoform in oocytes. These observations show that the P2X1 receptor is expressed in monocytes. Furthermore, these cells show no desensitizing currents at high ATP concentrations similar to those observed for homomeric PX1 and P2X1 *del* channels suggesting that this behavior could be mediates, at least in part for these P2X1 isoform, but not discarding the participation of PX7 receptor.

We performed assays with monocytes from the same subject where ATP induced different currents between cells tested. In monocyte one, ATP induced an inward current with fast desensitization kinetics similar to the reported for the homomeric P2X1 receptor, in the same monocyte a higher ATP concentration induced a response with different properties (Fig. 12b, upper panel). In monocyte two from the same donator the recorded current was similar to the P2X7 receptor properties (Fig. 12d, lower panel). These data are in agreement with our results showing heterogeneous expression of P2X receptors in monocytes and also differences in the response related to the ATP concentration applied (Fig. 12). Furthermore, these data suggest than the ATP stimulation could be sensed in a different way between monocytes on the same subject and that the elicited response is determined by the native P2X receptors expressed in each monocyte.

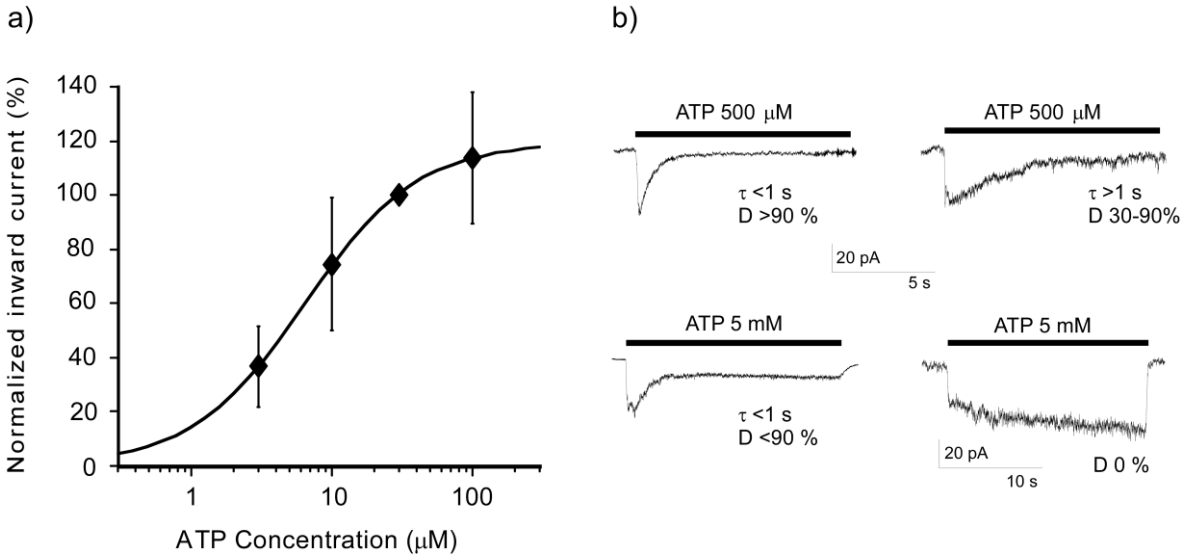

Figure 11. Currents mediated by wild type P2X1 receptors in human monocytes. Whole-cell patch clamp recordings were made from monocytes at a holding potential of -40 mV. Each symbol represents the average value of 4 to 9 experiments and the associated lines are S.E.M **a)** ATP concentration-response curve showing an EC_{50}=6.3±0.2 μM. **b)** Inward currents induced by different ATP concentrations in four different human monocytes. Note that currents have kinetics that resembles those observed in oocytes for P2X1 (see Fig. 9 and 10) and that some currents resemble the kinetics reported for homomeric P2X1 and P2X7 receptors activation.

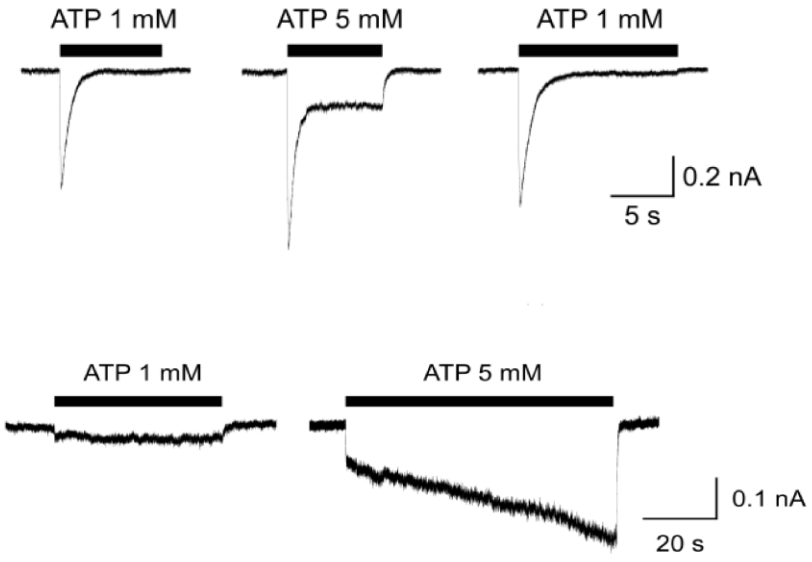

Figure 12. Different responses elicited by ATP stimulation in monocytes from the same subject. a) Recordings of representative currents induced by the application of ATP 1 y 5 mM in monocyte 1 (upper panel) or the monocyte 2 (lower panel). In monocyte one, ATP 1mM induced a fast inward current with fast desensitizing kinetics similar to the reported for the P2X1 subunit (upper pannel) ; whereas ATP 5 mM induced a fast inward current but with biphasic desensitizing kinetics . **b)** In monocyte two, the application of ATP 1mM evoked only a marginal current with no desensitizing kinetics, whereas the 5mM ATP application induced a fast inward current with non desensitizing kinetics and increases over time of the current amplitude that suggest the activation of P2X1*del* or P2X7 receptor (lower pannel). Currents were recorded at -40 mV.

9. DISCUSSION

This work shows that most human monocytes co-expressed two P2X1 receptor splicing variants which form functional homotrimeric channels when heterologously expressed. We also, for the first time, describe that currents mediated by P2X1 or P2X1*del* increased in amplitude when activated with high ATP concentrations in a similar fashion to those channels that increase their conductance under similar conditions, such as P2X7, P2X2, and P2X4 channels.

Our study shows that P2X1 receptor mRNA is present in 90% of human monocytes. Using Real Time PCR, expression of P2X1 receptor mRNA has been reported to be lower than P2X4 and P2X7 receptor mRNAs in human monocytes (Wang, Jacobsen et al. 2004). Such a study was carried out using groups of purified monocytes and did not offer any information regarding the percent of monocytes expressing this receptor, which we were able to determine by using single cell PCR. Furthermore, our observations demonstrate the expression of at least two receptor isoforms, the P2X1 canonical and its splice variant called P2X1*del* receptor, detected previously in platelets and megakaryocytic cells (Greco, Tonon et al. 2001). Both isoforms are co-expressed in most monocytes. As far as we know, this is the first time that P2X1*del* receptor is reported in human monocytes.

Previous studies have shown that the P2X1*del* receptor lacks 17 amino acids in the extracellular ligand-binding loop (Greco, Tonon et al. 2001) and it can be translated but fails to be exported to the cell membrane (Vial, Pitt et al. 2003). This

17 amino acids region comprises the amino acids 176 to 192, PALLREAENFTLFIKNS. P2X1*del* variant loses a NFT conserved glycosylation site, which has been related with the receptor incorporation to the plasmatic membrane (Lenertz, Wang et al. 2010) and with the receptor sensitivity for ATP (Roberts and Evans 2006). In addition, contradictory results exist regarding the functionality of the P2X1*del* variant, which was early reported to be preferentially activated by ADP in platelets and megakaryocytic cells (Greco, Tonon et al. 2001). A following report, however, did not find functional channels when P2X1*del* receptor was expressed (Vial, Pitt et al. 2003), in response to 100 μM ADP or ATP. Our results demonstrates that P2X1*del* receptors, when expressed in oocytes, can be assembled to form functional homomeric channels but with a very low ATP sensitivity, and also, P2X1*del* is translated in mammalian cells including blood cells. Because P2X1*del* receptors can be integrated into the cell membrane, we hypothesize that they might form heteromeric channels with other P2X receptors known to be expressed in monocytes, for instance the P2X1 (canonical), P2X4 and P2X7 receptors (Wang, Jacobsen et al. 2004). However, further studies are required to investigate the presence of these putative heteromeric channels. Evidence that P2X1 forms heteromeric channels with P2X4 have been published (Nicke, Kerschensteiner et al. 2005) and therefore, it is likely that any of the two P2X1 isoforms might form channels with P2X4 subunits.

Our data, from heterologous expression, show that currents mediated by homomeric P2X1 or P2X1*del* receptors increased in amplitude when activated with high ATP concentrations, in a similar fashion to those channels that increase their

conductance under similar stimulation, such as P2X7 (Surprenant, Rassendren et al. 1996; Gudipaty, Humphreys et al. 2001), P2X2 (Khakh, Bao et al. 1999; Virginio, MacKenzie et al. 1999), and P2X4 (Virginio, MacKenzie et al. 1999) channels. We hypothesize that P2X1 or P2X1*del* channels suffer a progressive dilation increasing single channel conductance. A similar behavior was observed with those currents mediated by native P2X receptors, in monocytes, suggesting that native P2X1 channel isoforms have the same behavior. However, monocytes also expressed P2X4 and P2X7 receptors (Wang, Jacobsen et al. 2004), which could be responsible for the secondary rise in current observed in monocytes.

Two P2X1 receptor splicing variants lacking the NFT consensus site have also been reported in rat tissues different to blood cells, the first one reported by Ohkubo (named P2X1a) was cloned form rat mesenteric artery and lacks 27 amino acids encoded by exon 6, this receptor fails to be expressed in the plasmatic membrane and to elicit an ATP induced response when expressed alone; the second splicing variant reported by Rangel-Yescas (named P2X1b) was cloned from rat optic nerve and lacks 27 aminoacids encoded by segments of exons 6 and 7, this receptor was also not functional as homomer. However, when both splicing variants were co-expressed with the wild type receptor, both were found in the plasmatic membrane, suggesting than this variants could associate with the wild type receptor to form heteromeric receptors (Ohkubo, Yamazaki et al. 2000; Rangel-Yescas, Vazquez-Cuevas et al. 2012).

Even when it was reported that this splicing variant was not found in HEK cell membranes due to poor traffic of the receptor, a similar variant named P2X1b

reported in rat and expressed in *Xenopus leavis* oocytes showed that this splicing variant could be found in the oocyte membrane even when is transfected alone, suggesting that P2X1*del* could be inserted in the plasmatic membrane in oocytes but not in HEK cells, and allowing us to record currents elicited by P2X1*del* receptor in oocytes, unfortunately for the rat splicing variant ATP concentrations higher than 100 uM never were tested (Rangel-Yescas, Vazquez-Cuevas et al. 2012). When both receptors were co-expressed, the responses showed the same fast desensitization component than the WT receptor but with lower current amplitude than the current obtained when the wild type receptor was expressed alone (fig 5c, 5d), this observation was also seen when the similar rat splicing variant P2X1b was expressed in oocytes. The dose-response curve showed less ATP sensitivity than P2X1wt (EC_{50} 4.65±1.34 uM), similar changes to ATP sensitivity have been reported when the NFT consensus was mutated, the P2X1 receptor mutant F185A showed an EC_{50} ten times higher than the P2X1*wt* receptor due to participation of the phenylalanine 185 in the ATP binding pocket (Roberts and Evans 2004) (Fig 5a). We do not have enough data to suggest that P2X1*wt* and P2X1*del* could be associated to form heteromeric receptors in oocytes, however our data show evidence of P2X1*del* participation in currents recorded in oocytes co-expressing both receptors. In these oocytes the current amplitude increased more than 100% when concentrations higher than 1000 uM were applied, as seen in oocytes expressing P2X1*del* homomeric receptors, and unlike oocytes expressing P2X1*wt* homomeric receptors in which the maximal amount of current was reached with ATP 1000 μM. Our data suggest that P2X1*del* could be regulating the P2X1*wt* receptor since their co-expression decreases the current

amplitude, the regulation process could involve altered transport mechanisms to the membrane or the replacement of P2X1*wt* for P2X1*del* in the membrane. Other groups have reported that P2X1 is involved in apoptosis and activation of transcription factors in cells different to monocytes, but the role of P2X1 receptor in monocytes remains to be determined.

We performed flow cytometry experiments to determine the expression of P2X1 protein, we found the expression of the P2X1 in a high percentage of monocytes, the percent of cells expressing the protein is in agreement with the percent of cells expressing the receptor mRNA. We were unable to describe if the expression was located in the membrane since the antibody used is directed to c-terminus of the receptor, we also were unable to distinguish between the wild type and the splicing variant with the antibody used since the lack sequence is located in the extracellular domain. P2X1 receptor protein was found in a higher percent of the non classical monocytes (CD14+ CD16+) versus the classical monocytes (CD14+ CD16-); this finding is consistent with the work of Wong, who reported an increase in the P2X1 receptor mRNA in the non classical monocytes versus classical monocytes by analyzing the gene expression profile in monocyte subsets (Wong, Tai et al. 2011).

It has been reported a regulation in the P2X subunit expression in monocytes in the process of differentiation to macrophages, as well as monocyte subpopulations are different maturation stages in the same process (Gudipaty, Humphreys et al. 2001; Gordon and Taylor 2005; Ziegler-Heitbrock 2007). Therefore, the differences in P2X1 receptor expression among monocyte subsets could be related to the

differentiation process from monocytes to macrophages, suggesting the expression of P2X1 receptor in a higher number of macrophages.

We also found the expression of P2X4 in a high number of monocytes, co-expressed in the 70% of the monocytes analyzed with both P2X subunits. In some cells, ATP elicited an inward current with a slow desensitization component, similar to the reported for P2X4 homomeric receptors. It has been reported that P2X1 and P2X4 receptors could form heteromeric receptors, however further investigation is needed to determine if that happens in monocytes. P2X4 protein was detected in a lower percentage of monocytes, however P2X4 mRNA was found in 85% of the cells analyzed. On the other hand, P2X7 is the most commonly reported P2X receptor in monocytes and macrophages, we were unable to find its mRNA and proteins in the monocytes analyzed, maybe due to the low differentiation stage of monocytes. ATP induced currents showed characteristics that suggest the expression and activation of P2X7, but we did not find the receptor protein, suggesting that the currents with no desensitization could be attributed to the expression and activation of P2X1*del.* Our single cell analysis showed heterogeneous expression of P2X subunits in monocytes, this could imply that the response elicited by ATP is different among monocytes depending on the subunits that each monocyte expresses. The expression of P2X receptors could also be further investigated since the expression pattern for P2X receptors could be related with different monocyte subpopulations or activation signaling.

10. CONCLUSION

This study demonstrates, for the first time, the expression of P2X1 receptor and its splicing variant P2X1*del* in most human monocytes and describes functional homomeric P2X1*del* channels, which, together with the canonical P2X1, show a secondary rise in current, similar to that described for P2X2, P2X4 and P2X7 channels. The mechanism and relevance for this secondary rise in current and the role of P2X1 receptor isoforms in monocytes remains to be determined. In addition, we show that human monocytes have a heterogeneous expression of P2X receptors as P2X1 and P2X7 among monocytes subpopulations, which could be reflected in a variety of responses elicited by ATP.

Table 1. Primers used in RT-PCR and Single Cell-PCR.

Primer	PCR	Alignment Temperature (°C)	Sequence
X1-F1	**RT-PCR/1st**	**60**	**S** 5’ CCCCCAGAAGCTCTACCAT 3’
X1-R1	**RT-PCR/1st**	**60**	**AS** 5’ TGCACCCAGTCAGGAGTT 3’
X1-F2	**2nd**	**56**	**S** 5’ CGGGTGGGTGTTTCTCTATG 3’
X1-R2	**2nd**	**56**	**AS** 5’ CCAACCACTCCACCCTTCTC 3’
X1-F3	**2nd**	**60**	**S** 5’ GACATCCCGCGCATCAGC 3’
X7-F1	**RT-PCR**	**55**	**S** 5' AGCAAAGGAATCCAGACCGGCAG 3'
X7-R1	**RT-PCR**	**55**	**AS** 5' AAGAGCTCGGAGGTGGTGATGCA 3'
X7-F2	**1st**	**55**	**S** 5’ AAGCTTGCCCTGTCAGGAAGAGT 3’
X7-F3	**2nd**	**55**	**S** 5’ AAGAGGAGATCGTGGAGAATGGA 3’
X7-R2	**1st , 2nd**	**55**	**AS** 5’ ATATGGGAGCGACAGCAGTTA 3’
X4-F1	**1st**	**58**	**S** 5’ AAGCTTCAGACCGACTAGGGGACT 3’
X4-R1	**1st**	**58**	**AS** 5’ CTCGAGTGGAGTGGAGACTCAATCAG 3’
X4-F2	**RT-PCR/2nd**	**50**	**S** 5' TTCCTGTTCGAGTACGA 3'
X4-R2	**RT-PCR/2nd**	**50**	**AS** 5' CTGTAGTACTTGGCAAAC 3'
X4-F3	**3th**	**57**	**S** 5’ CGTTACGACCAAGGTCAAGGG 3’
X4-R3	**3th**	**57**	**AS** 5’ TGGAAACTGTGTCCTGCGTTC 3’

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APPENDIX A

Cintya López-López, Josue Jaramillo-Polanco, Diana P. Portales-Pérez, Karen S. Gómez-Coronado, Jessica G. Rodríguez-Meléndez, Juan D. Cortés-García, Rosa Espinosa-Luna, Luis M. Montaño, and Carlos Barajas-López**. Two P2X1 receptor transcripts able to form functional channels are present in most human monocytes.** Accepted on the European Journal of Pharmacology.

Two P2X1 receptor transcripts able to form functional channels are present in most human monocytes

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Abstract

To characterize the presence and general properties of P2X1 receptors in single human monocytes we used RT-PCR, flow cytometry, and the patch-clamp and the two-electrode voltage-clamp techniques. Most human monocytes expressed the canonical P2X1 and its splicing variant P2X1*del* mRNAs. P2X1 receptor immunoreactivity was observed in most monocytes. Currents mediated by P2X1 (EC_{50}=1.9±0.8 μM) and P2X1*del* (EC_{50} >1000 μM) channels, expressed in *Xenopus leavis oocytes*, have different ATP sensitivity and kinetics. Both currents mediated by P2X1 and P2X1*del* channels kept increasing during the continuous presence of high ATP concentrations. Currents mediated by the native P2X1 receptors in human monocytes showed an EC_{50}=6.3±0.2 μM. Currents have kinetics that resemble those observed for P2X1 and P2X1*del* receptors in oocytes. Our study is the first to demonstrate the expression of P2X1 transcript and its splicing variant P2X1*del* in most human monocytes. We also, for the first time, described functional homomeric P2X1*del* channels and demonstrated that currents mediated by P2X1 or P2X1*del* increased in amplitude when activated with high ATP concentrations in a similar fashion to those channels that increase their conductance under similar conditions, such as P2X7, P2X2, and P2X4 channels.

Keywords: Heterologous expression, P2X receptors, Blood cells, Patch clamp, P2X1*del* receptors

APPENDIX B

Cortés-Garcia JD, **López-López C**, Cortez-Espinosa N, García-Hernández MH, Guzmán-Flores JM, Layseca-Espinosa E, Portales-Cervantes L, Portales-Pérez DP. **Evaluation of the expression and function of the P2X7 receptor and ART1 in human regulatory T-cell subsets.** Immunobiology. 2016 Jan;221(1):84-93. doi: 10.1016/j.imbio.2015.07.018. Epub 2015 Jul 29.

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Evaluation of the expression and function of the P2X7 receptor and ART1 in human regulatory T-cell subsets

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ABSTRACT

Regulatory T cells that express CD39 (CD39+ Treg) exhibit specific immunomodulatory properties. Ectonucleotidase CD39 hydrolyses ATP and ADP. ATP is a ligand of the P2X7 receptor and induces the shedding of CD62L and apoptosis. However, the role of ATP in CD39+ Treg cells has not been defined. Furthermore, NAD can activate the P2X7 receptor via ADP-ribosyltransferase (ART) enzymes and cause cell depletion in murine models. We evaluated the expression and function of P2X7 and ART1 in CD39+ Treg and CD39- Treg cells in the presence or absence of ATP and NAD. We isolated peripheral blood mononuclear cells from healthy subjects and purified CD4+ T cells, CD4+ CD25+ T cells and CD4+ CD25+ CD39+ T cells. P2X7 and ART1 expression was assessed by flow cytometry and real-time PCR. Our results showed low P2X7 expression on CD39+ Treg cells and higher levels of ART1 expression in CD4+ CD39+ T cells than the other subtypes studied. Neither shedding of CD62L nor cell death of CD39+ Treg or CD39- Treg cells was observed by 1 mM ATP or 60 μM NAD. In contrast, P2Xs receptor-dependent proliferation with 300 μM ATP, was inhibited by NAD in the different cell types analysed. The NAD proliferation-inhibition was increased with P2Xs and A2a agonist and was reversed with P2Xs and A2a antagonist, therefore NAD inhibits P2Xs-dependent proliferation and A2a activation. In conclusion, our results suggest that the altered function and expression of P2X7 and ART1 in the human CD39+ Treg or CD39− Treg cells could participate in the resistance against cell death induced by ATP or NAD.

APPENDIX C

De Ita M, Vargas MH, Carbajal V, Ortiz-Quintero B, **López-López C**, Miranda-Morales M, Barajas-López C, Montaño LM. **ATP releases ATP or other nucleotides from human peripheral blood leukocytes through purinergic P2 receptors**. Life Sci. 2016 Jan 15;145:85-92. doi: 10.1016/j.lfs.2015.12.013. Epub 2015 Dec 8.

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ATP releases ATP or other nucleotides from human peripheral blood leukocytes through purinergic P2 receptors

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ABSTRACT

Aims: Almost every eukaryotic cell releases ATP under certain conditions. The idea that ATP induces the release of ATP has been scantly investigated.
Methods: We explored this possibility by assessing the rate of exogenous ATP breakdown (measured by phosphates production) by human peripheral blood leukocytes. The role of P2Y and P2X receptors was evaluated pharmacologically, by patch clamp, or by flow cytometry.
Key findings: In mononuclear and/or polymorphonuclear cells, ATP increased phosphates formation in a time- and concentration-dependent manner. Uncoupling of P2Y receptors with N-ethylmaleimide and antagonism of P2Y and P2X receptors through suramin reduced phosphate formation after 500 μM ATP, suggesting that part of the phosphate production was due to activation of P2 receptors, with subsequent release of ATP or other nucleotides. Similar results were obtained with UTP and ATPγS. Gadolinium (connexins inhibitor) also significantly reduced the ATP-induced phosphate production. Blockade of P2X receptors with SKF 96365 or NF023 did not modify the phosphate production. In monocytes, 500 μM ATP induced inward currents suggestive of $P2X_1$ activation, but higher concentrations (1–5 mM) induced inward currents suggestive of $P2X_7$ activation. We discarded a role of adenosine in the ATP-evoked nucleotides release. Flow cytometry identified that almost all mononuclear and polymorphonuclear cells expressed $P2Y_{1,2,4,6,11}$ receptors.
Significance: 500 μM ATP induced the release of ATP or other nucleotides through activation of $P2Y_{2,4,6,11}$ receptors in human leukocytes, and probably via P2X receptors at higher concentrations. This ATP-induced nucleotides release constitutes a potential mechanism leading to amplification of ATP signaling.