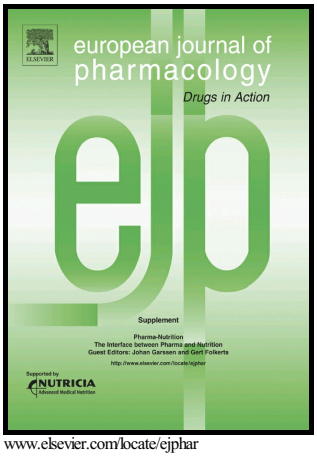

www.elsevier.com/locate/ejphar

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

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, Carlos Barajas-López

PII: S0014-2999(16)30678-1
DOI: http://dx.doi.org/10.1016/j.ejphar.2016.10.033
Reference: EJP70899

To appear in: *European Journal of Pharmacology*

Received date: 22 August 2016
Revised date: 18 October 2016
Accepted date: 27 October 2016

Cite this article as: 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 Carlo Barajas-López, Two P2X1 receptor transcripts able to form functional channel are present in most human monocytes, *European Journal of Pharmacology* http://dx.doi.org/10.1016/j.ejphar.2016.10.033

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

Cintya López-López[1], Josue Jaramillo-Polanco[1], Diana P. Portales-Pérez[2], Karen S. Gómez-Coronado[1], Jessica G. Rodríguez-Meléndez[1], Juan D. Cortés-García[2], Rosa Espinosa-Luna[1], Luis M. Montaño[3], Carlos Barajas-López[1*]

[1]Instituto Potosino Investigación Científica y Tecnológica, Camino a la Presa San José 2055. Col. Lomas 4ª Sección, CP. 78216, San Luis Potosí. México.

[2]Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, SLP, México

[3]Departamento de Farmacología, Facultad de Medicina, Universidad Nacional Autónoma de México, México DF, México

[*]**Corresponding autor**. Instituto Potosino de Investigación Científica y Tecnológica (IPICYT) Camino a la Presa San José 2055 Col. Lomas 4a Sección San Luis Potosí, SLP, CP78216, México Tel: +52(444) 834 2035 fax: +52(444) 834-2010 cbarajas@ipicyt.edu.mx

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 (90%) and its splicing variant P2X1del (88%) mRNAs. P2X1 receptor immunoreactivity was also observed in 70% of these cells . Currents mediated by P2X1 (EC_{50}=1.9±0.8 μM) and P2X1del (EC_{50} >1000 μM) channels, expressed in Xenopus

leavis oocytes, have different ATP sensitivity and kinetics. Both currents mediated by P2X1 and P2X1del 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 P2X1del receptors in oocytes. Our study is the first to demonstrate the expression of P2X1 transcript and its splicing variant P2X1del in most human monocytes. We also, for the first time, described functional homomeric P2X1del channels and demonstrated that currents mediated by P2X1 or P2X1del receptors, during heterologous expression, 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, P2X1del receptors

1. Introduction

Human monocytes represent 10% of the nucleated blood cells and their most prominent function is as a systemic reservoir for the renewal of tissue macrophages (Yona and Jung, 2010). They are divided on the basis of surface CD14 and CD16 expression (Ziegler-Heitbrock, 2015), in classical ($CD14^{++}/CD16^{-}$) intermediate ($CD14^{-}/CD16^{+}$) and non-classical ($CD14^{+}/CD16^{++}$). Classical monocytes are most prevalent and also more phagocytic than the non-classical. Intermediate monocytes express HLA and CD74 in higher levels, which makes them antigen presenter cells. This subset also produce high levels of reactive oxigen species, IL-1β and TNFα (Serbina et al., 2008; Heine et al.,

2012). The non-classical cells are pro-inflammatory, constitute 20% of circulating monocytes, produce pro-inflammatory cytokines, and are preferentially mobilized during inflammation (Strauss-Ayali et al., 2007; Auffray et al., 2009; Wong et al., 2012).

ATP reaches the extracellular space by different mechanisms (Bodin and Burnstock, 2001; Chekeni et al., 2010; Sridharan et al., 2010; Orellana et al., 2011; Lohman et al., 2012; De Ita et al., 2016), where it can activate metabotropic (P2Y) and ionotropic (P2X) receptors expressed by immune cells (Di Virgilio et al., 2001; De Ita et al., 2016). ATP is released by platelets (Beigi et al., 1999), lymphocytes (Schenk et al., 2008; Yip et al., 2009), or other cells (Montalbetti et al., 2011). Activation of P2 receptors triggers cytokine release and regulates immune responses, including: shedding of membrane antigens as CD62L, activation of transcription factors related to inflammation, and cell proliferation (Ferrari et al., 1997; Burnstock and Knight, 2004; Cauwels et al., 2014).

There are seven P2X subunits (P2X1-P2X7), encoded by different genes, which can form trimeric channels permeable to cations (North, 2002). In human macrophages, the P2X7 receptor activation has been implicated in the killing of mycobacteria, activation of TNFα, inflammasome activation and release of the interleukin IL-1β, as well as cytotoxic actions (Lammas et al., 1997; Ferrari et al., 1999; Suzuki et al., 2004) but little is known about the other P2X subunits participation in these events. However, mouse peritoneal macrophages display whole cell currents mediated by P2X1 and P2X4 homomeric receptor activation (Sim et al., 2007; Kessler et al., 2011). P2X1 is a widely distributed receptor in blood cells and has been associated with platelet aggregation (Oury et al., 2003). Human monocytes also express both P2X and P2Y receptors in particular, subunits P2X1, P2X4 and P2X7 mRNAs (Wang et al., 2004; De Ita et al.,

2016). Therefore, the aim of this work was to investigate the distribution of P2X1 receptor transcripts in human monocytes. We showed that most monocytes co-express P2X1 and a splicing variant named P2X1del, both of which can form functional homomeric channels when they were heterologously expressed.

2. Material and methods

2.1 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from 4 ml of venous blood from healthy donators 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 700 g for 20 min. 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 h. 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 h after cell plating.

2.2 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 ml 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 subunits was performed by nested PCR, using the following primers and conditions; for the first P2X1 PCR we used the primers pair F1 + R1 obtaining a 1.33 kb PCR product. For nested PCR 1 µl of the first PCR was used as template and using primers F2 + R2 we obtained a 627 pb and a 576 pb fragments. For the specific amplification of the splicing variant we performed another nested PCR using 1 µl of the first reaction and primers F3 + R2 obtaining a PCR product of 197 pb (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, 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. 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.

2.3 Flow cytometry analysis of P2X1 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. 2A). In the rest of experiments, PBMC were incubated with mouse antihuman CD14 FITC labeled antibody for 20 min (BD Pharmigen) in the dark and also fixed and permeabilized as described above (see Fig 2B). One set of PBMC incubated with CD14 antibody was also incubated with rabbit anti-human P2X1 polyclonal antibody (Alomone labs, Israel) directed to the receptor C-terminus, corresponding to amino acids 382-399 of rat P2X1, which was added for 30 min at 4°C in the dark. These 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. To investigate specificity of the primary anti-P2X1 antibody, we also carried out experiments without this antibody (see Fig. 2C). Cells were fixed into 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).

2.4 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 (21-25°C). Patch pipettes were made as previously described (Barajas-Lopez 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; $CaCl_2$, 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 adjusted 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).

2.5 Cloning of P2X1 receptors

PCR was performed using human P2X1 primers designed at the 5' and 3' UTRs regions to amplify the entire coding sequence including the Kosak sequence, from the first single cell PCR reaction as previously described. The primers used were: forward primer (F) 5'-**GGATCC**AGCCGGCCCACCATGG-3'; and reverse primer (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 sub cloned in pCDNA3 vector. The nucleotide sequences were confirmed by a minimal of three sequencing rounds using different primers.

2.6 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 of cap and poli-A mRNA (P2X1 and P2X1del, alone) and incubated at 14°C for 12-36 h before the electrophysiological experiments. The cap and poli-A 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 s or until the current reach a peak, and then washed off for 5 min. ATP solutions were freshly prepared and maintained in ice to decrease degradation. The recording chamber was continuously superfused with standard external solution (NaCl, 88 mM; KCl, 2 mM; $CaCl_2$, 1 mM; $MgCl_2$, 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).

2.7 Data analysis

In each oocytes, we normalized I_{ATP} considering the response to ATP 100 μM as 100% for P2X1 and the response to ATP 5 mM for P2X1del, 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 with a three parameters logistic function (Kenakin, 1993) using KaleidaGraph 4.1.0 (Synergy Software).

3. Results

3.1 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. 1A). Using oligonucleotides F1 and R1 (Table 1) we obtained a PCR product of 1.33 Kb (Fig. 1B). 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 pb corresponding to the first part of the exon 6. The sequence corresponded to a splicing variant previously reported (Greco et al., 2001), expressed in human platelets and named P2X1del (Fig. 1A). 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 F2 + R2 (Table 1) we were able to amplify both, the P2X1 mRNA in 90% of the monocytes analyzed (7 healthy subjects, 54 cells, Figure 1C, 1D), whereas, P2X1del was found in 88% of the monocytes (4 healthy subjects, 36 cells), the later average was obtained from cells in which the presence of P2X1del expression was confirmed using the F3 oligonucleotide (Table 1, Fig. 1A) 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 pb PCR product was confirmed by sequencing (Fig. 1A-C). In the same 36 monocytes tested for both mRNAs we found P2X1 in 34 and co-expressed P2X1del in 31 cells (86%; Fig.1D). P2X1 receptor transcripts were found in monocytes from all subjects tested.

3.2 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 (Fig 2B) 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 subunit and revealed with an APC secondary antibody (Fig. 2D). We found that 70±4 % (11 different subjects) of $CD14^+$ monocytes, expressed P2X1 receptor protein (Fig. 2E-F). The lost region of P2X1del is extracellular and, therefore, most likely this antibody does not distinguish between the two isoforms, as previously shown (Greco et al., 2001). The percent of monocytes expressing P2X1 protein is lower than those expressing the P2X1 receptor mRNA, which could be the result of a higher rate of false negatives observations with flow cytometry or due to the fact that mRNA levels do not predict perfectly protein expression in monocytes, as it has been shown previously (Guo et al., 2008).

3.3 Heterologous expression of P2X1 or P2X1del mRNAs

Expression of P2X1 or P2X1del mRNAs in Xenopus Leavis oocytes formed functional homomeric channels. P2X1 receptor showed the previously reported characteristics of P2X1 homomeric receptor (North, 2002): high ATP sensitivity (EC_{50}=1.9±0.8 μM), activation at low ATP concentrations (0.1 μM), a maximal peak current at 10-30 μM ATP (Fig. 3A), a fast desensitization kinetics (Tau 0.65±0.12 s, n=4, Fig. 3B and 3D), and a large percent of desensitization (Fig. 3D). At higher ATP concentrations (3-5 mM), the current amplitude of the canonical receptor continued increasing after the fast desensitization component (Fig. 3E). P2X1del receptor was unresponsive to low ATP concentrations (≤100 μM, Fig. 3A) 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. 3C). We did not see any fast transient current. Indeed,

P2X1del responses did not show desensitization and when exposed to 3 and 5 mM ATP, the current amplitude increased under the continuous stimulation (Fig. 3C), similarly to what happens with the canonical P2X1 receptor at these high concentrations (Fig 3E). However, not inward current was observed, even by high ATP concentrations (3-5 mM), in 8 oocytes injected with control solution without any mRNA (Fig. 3C).

3.4 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 panel of Fig 4B), 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 4A, with an EC_{50} of 6.3±0.2 μM, this value was significantly larger ($P<0.001$, unpaired Student's *t*-test, two tails) than that observed in oocytes expressing the P2X1 homomeric receptors (EC_{50}=1.9±0.8 μM). At high ATP concentrations (≥ 1 mM), current amplitude was kept increasing during the agonist exposure, some times after a fast desensitizing current (Fig. 4B, middle panel) or right after the current onset (Fig. 4B, lower panel), similar to what it was observed for both P2X1 isoforms in oocytes. These observations show that the P2X1 receptor is expressed in monocytes. Furthermore, these cells show not desensitizing currents at high ATP concentrations, similar to those observed for homomeric P2X1 and P2X1del channels suggesting that this behavior could be mediated, at least in part for these

P2X1 isoforms.

4. Discussion

This work shows that most human monocytes co-expressed two P2X1 receptor transcripts, which form functional homomeric channels when are heterologously expressed. We also, for the first time, describe that currents mediated by P2X1 or P2X1del 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 both P2X1 receptor mRNAs are present in about 85% 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 et al., 2004). Such a study was carried out using groups of purified monocytes and did not offer any information regarding the percent of cells 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 P2X1del receptor, found to be present and translated in platelets and megakaryocytic cells (Greco et al., 2001). Both mRNA variants are co-expressed in most monocytes. As far as we know, this is the first time that P2X1del receptor mRNA is reported in human monocytes.

Previous studies have shown that the P2X1del receptor lacks 17 amino acids in the extracellular ligand-binding loop (Greco et al., 2001) and it can be translated but fails to be exported to the cell membrane (Vial et al., 2003). This 17 amino acids region comprises the amino acids 176 to 192, PALLREAENFTLFIKNS. P2X1del variant loses a NFT conserved glycosylation site, which has been related with the receptor

incorporation to the plasmatic membrane (Lenertz et al., 2010) and with the receptor sensitivity for ATP (Roberts and Evans, 2006). In addition, contradictory results exist regarding the functionality of the P2X1del variant, which was early reported in platelets and megakaryocytic cells and to be preferentially activated by ADP (Greco et al., 2001). A following report, however, did not find functional channels when P2X1del receptor was expressed (Vial et al., 2003), in response to 100 μM ADP or ATP. Our results demonstrate that P2X1del receptors, when expressed in oocytes, can be assembled to form functional homomeric channels and P2X1del mRNA is also translated in mammalian cells including blood cells (Greco et al., 2001), we hypothesized 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 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 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 P2X1del 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 et al., 1996; Gudipaty et al., 2001), P2X2 (Khakh et al., 1999; Virginio et al., 1999), and P2X4 (Virginio et al., 1999) channels. We hypothesize that P2X1 or P2X1del 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 and they might contribute to the current

increase in these cells when high concentrations are used. However, monocytes also expressed P2X4 and P2X7 receptors (Wang et al., 2004), which could also contribute to the secondary rise in current observed in monocytes.

In conclusion, this study demonstrates for the first time, the expression of P2X1 transcript and its splicing variant P2X1del in most human monocytes, describes that homomeric P2X1del channels are functional, and reports that both P2X1 isoforms 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 remain to be determined.

Acknowledgments

This work was supported by CONACYT, México (Projects Num. 134687 and 219859). Scholarships of CL-L, JJ-P, JDC-G, and JR-M were supported by CONACYT.

References

Auffray, C., Sieweke, M.H.Geissmann, F., 2009. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol 27, 669-692.

Barajas-Lopez, C., Huizinga, J.D., Collins, S.M., Gerzanich, V., Espinosa-Luna, R.Peres, A.L., 1996. P2x-purinoceptors of myenteric neurones from the guinea-pig ileum and their unusual pharmacological properties. Br J Pharmacol 119, 1541-1548.

Beigi, R., Kobatake, E., Aizawa, M.Dubyak, G.R., 1999. Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. Am J Physiol 276, C267-278.

Bodin, P. Burnstock, G., 2001. Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. J Cardiovasc Pharmacol 38, 900-908.

Burnstock, G. Knight, G.E., 2004. Cellular distribution and functions of P2 receptor subtypes in different systems. Int Rev Cytol 240, 31-304.

Cauwels, A., Rogge, E., Vandendriessche, B., Shiva, S.Brouckaert, P., 2014. Extracellular ATP drives systemic inflammation, tissue damage and mortality. Cell Death Dis 5, e1102.

Chekeni, F.B., Elliott, M.R., Sandilos, J.K., Walk, S.F., Kinchen, J.M., Lazarowski, E.R., Armstrong, A.J., Penuela, S., Laird, D.W., Salvesen, G.S., Isakson, B.E., Bayliss, D.A.Ravichandran, K.S., 2010. Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature 467, 863-867.

De Ita, M., Vargas, M.H., Carbajal, V., Ortiz-Quintero, B., Lopez-Lopez, C., Miranda-Morales, M., Barajas-Lopez, C.Montano, L.M., 2016. ATP releases ATP or other nucleotides from human peripheral blood leukocytes through purinergic P2 receptors. Life Sci 145, 85-92.

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

Ferrari, D., Los, M., Bauer, M.K., Vandenabeele, P., Wesselborg, S.Schulze-Osthoff, K., 1999. P2Z purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. FEBS Lett 447, 71-75.

Ferrari, D., Wesselborg, S., Bauer, M.K.Schulze-Osthoff, K., 1997. Extracellular ATP activates transcription factor NF-kappaB through the P2Z purinoreceptor by selectively targeting NF-kappaB p65. J Cell Biol 139, 1635-1643.

Greco, N.J., Tonon, G., Chen, W., Luo, X., Dalal, R.Jamieson, G.A., 2001. Novel structurally altered P(2X1) receptor is preferentially activated by adenosine diphosphate in platelets and megakaryocytic cells. Blood 98, 100-107.

Gudipaty, L., Humphreys, B.D., Buell, G.Dubyak, G.R., 2001. Regulation of P2X(7) nucleotide receptor function in human monocytes by extracellular ions and receptor density. Am J Physiol Cell Physiol 280, C943-953.

Guo, Y., Xiao, P., Lei, S., Deng, F., Xiao, G.G., Liu, Y., Chen, X., Li, L., Wu, S., Chen, Y., Jiang, H., Tan, L., Xie, J., Zhu, X., Liang, S.Deng, H., 2008. How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. Acta Biochim Biophys Sin (Shanghai) 40, 426-436.

Heine, G.H., Ortiz, A., Massy, Z.A., Lindholm, B., Wiecek, A., Martinez-Castelao, A., Covic, A., Goldsmith, D., Suleymanlar, G., London, G.M., Parati, G., Sicari, R., Zoccali, C.Fliser, D., 2012. Monocyte subpopulations and cardiovascular risk in chronic kidney disease. Nat Rev Nephrol 8, 362-369.

Kenakin, R., 1993. Pharmacologic Analysis of Drug–receptor Interaction. 2nd Ed. Raven Press, Ltd., New York, N.Y.

Kessler, S., Clauss, W.G., Gunther, A., Kummer, W.Fronius, M., 2011. Expression and functional characterization of P2X receptors in mouse alveolar macrophages. Pflugers Arch 462, 419-430.

Khakh, B.S., Bao, X.R., Labarca, C.Lester, H.A., 1999. Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds. Nat Neurosci 2, 322-330.

Lammas, D.A., Stober, C., Harvey, C.J., Kendrick, N., Panchalingam, S.Kumararatne, D.S., 1997. ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. Immunity 7, 433-444.

Lenertz, L.Y., Wang, Z., Guadarrama, A., Hill, L.M., Gavala, M.L.Bertics, P.J., 2010. Mutation of putative N-linked glycosylation sites on the human nucleotide receptor P2X7 reveals a key residue important for receptor function. Biochemistry 49, 4611-4619.

Lohman, A.W., Billaud, M.Isakson, B.E., 2012. Mechanisms of ATP release and signalling in the blood vessel wall. Cardiovasc Res 95, 269-280.

Montalbetti, N., Leal Denis, M.F., Pignataro, O.P., Kobatake, E., Lazarowski, E.R.Schwarzbaum, P.J., 2011. Homeostasis of extracellular ATP in human erythrocytes. J Biol Chem 286, 38397-38407.

Nicke, A., Kerschensteiner, D.Soto, F., 2005. Biochemical and functional evidence for heteromeric assembly of P2X1 and P2X4 subunits. J Neurochem 92, 925-933.

North, R.A., 2002. Molecular physiology of P2X receptors. Physiol Rev 82, 1013-1067.

Orellana, J.A., Froger, N., Ezan, P., Jiang, J.X., Bennett, M.V., Naus, C.C., Giaume, C.Saez, J.C., 2011. ATP and glutamate released via astroglial connexin 43 hemichannels mediate neuronal death through activation of pannexin 1 hemichannels. J Neurochem 118, 826-840.

Oury, C., Kuijpers, M.J., Toth-Zsamboki, E., Bonnefoy, A., Danloy, S., Vreys, I., Feijge, M.A., De Vos, R., Vermylen, J., Heemskerk, J.W.Hoylaerts, M.F., 2003. Overexpression of the platelet P2X1 ion channel in transgenic mice generates a novel prothrombotic phenotype. Blood 101, 3969-3976.

Roberts, J.A. Evans, R.J., 2006. Contribution of conserved polar glutamine, asparagine and threonine residues and glycosylation to agonist action at human P2X1 receptors for ATP. J Neurochem 96, 843-852.

Schenk, U., Westendorf, A.M., Radaelli, E., Casati, A., Ferro, M., Fumagalli, M., Verderio, C., Buer, J., Scanziani, E.Grassi, F., 2008. Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. Sci Signal 1, ra6.

Serbina, N.V., Jia, T., Hohl, T.M.Pamer, E.G., 2008. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol 26, 421-452.

Sim, J.A., Park, C.K., Oh, S.B., Evans, R.J.North, R.A., 2007. P2X1 and P2X4 receptor currents in mouse macrophages. Br J Pharmacol 152, 1283-1290.

Sridharan, M., Adderley, S.P., Bowles, E.A., Egan, T.M., Stephenson, A.H., Ellsworth, M.L.Sprague, R.S., 2010. Pannexin 1 is the conduit for low oxygen tension-induced ATP release from human erythrocytes. Am J Physiol Heart Circ Physiol 299, H1146-1152.

Strauss-Ayali, D., Conrad, S.M.Mosser, D.M., 2007. Monocyte subpopulations and their differentiation patterns during infection. J Leukoc Biol 82, 244-252.

Surprenant, A., Rassendren, F., Kawashima, E., North, R.A.Buell, G., 1996. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Science 272, 735-738.

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

Vial, C., Pitt, S.J., Roberts, J., Rolf, M.G., Mahaut-Smith, M.P.Evans, R.J., 2003. Lack of evidence for functional ADP-activated human P2X1 receptors supports a role for ATP during hemostasis and thrombosis. Blood 102, 3646-3651.

Virginio, C., MacKenzie, A., Rassendren, F.A., North, R.A.Surprenant, A., 1999. Pore dilation of neuronal P2X receptor channels. Nat Neurosci 2, 315-321.

Wang, L., Jacobsen, S.E., Bengtsson, A.Erlinge, D., 2004. P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. BMC Immunol 5, 16.

Wong, K.L., Yeap, W.H., Tai, J.J., Ong, S.M., Dang, T.M.Wong, S.C., 2012. The three human monocyte subsets: implications for health and disease. Immunol Res 53, 41-57.

Yip, L., Woehrle, T., Corriden, R., Hirsh, M., Chen, Y., Inoue, Y., Ferrari, V., Insel, P.A.Junger, W.G., 2009. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. FASEB J 23, 1685-1693.

Yona, S. Jung, S., 2010. Monocytes: subsets, origins, fates and functions. Curr Opin Hematol 17, 53-59.

Ziegler-Heitbrock, L., 2015. Blood Monocytes and Their Subsets: Established Features and Open Questions. Front Immunol 6, 423.

Figure 1. Most human monocytes express P2X1 and P2X1del mRNAs. Using RT-

PCR and Single Cell PCR, we amplified the complete ORF of P2X1 subunit and a splicing variant P2X1, which lacks 51 pb at the exon 6 in monocytes (P2X1del). **A,** Schematic representation of the exon-organization of P2X1 and P2X1del mRNAs and primer locations (F1, F2, R1-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 (F2 + R2) or P2X1del (F3 + 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 and co-expressing P2X1 transcripts.

Figure 2. P2X1 receptor protein is expressed in most human monocytes. Peripheral Blood Mononuclear cells (PBMC) from healthy subjects were co-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) rectangle. 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 materials and methods. Cells co-expressing CD14+ P2X1 proteins are indicated by the

upper right (blue) rectangle. **E,** Histogram of P2X1+ cells in the monocyte gate. **F,** Percentage of cells expressing P2X1 receptors in CD14+ cells from 11 healthy subjects.

Figure 3. Properties of currents mediated by P2X1 and P2X1del channels heterologously expressed in Xenopus Leavis oocytes. A, Concentration-response curves for receptors: P2X1 (EC_{50}=1.9±0.8 μM) and P2X1del (EC_{50} >1000 μM). Currents were measured at the peak for P2X1, however, for P2X1del currents were measured 10 s after ATP addition, and normalized against responses to 100 μM and 5 mM, respectively. 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 P2X1del receptors are activated only at high ATP concentrations and show not desensitization; indeed, current amplitudes keep increasing during the continuous presence of ATP. **D,** Desensitization of P2X1 is well fitted with one exponential function (left panel), also the receptor showed a high percentage of desensitization (right panel). **E,** Inward currents induced by high ATP concentrations in oocytes expressing P2X1 receptors. Oocytes were clamped at -60 mV.

Figure 4. Currents mediated by native P2X 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 three different human

monocytes. Note that currents have kinetics that resembles those observed in oocytes for P2X1 (see Fig. 3).

Table 1. Sequences of the sense (S) and antisense (AS) oligonucleotides used in the RT-PCR amplifications

Primer	PCR	Alignment Temperature (°C)	Sequence
F1	1st	60	S 5' CCCCCAGAAGCTCTACCAT 3'
R1	1st	60	AS 5' TGCACCCAGTCAGGAGTT 3'
F2	2nd	56	S 5' CGGGTGGGTGTTTCTCTATG 3'
R2	2nd	56	AS 5' CCAACCACTCCACCCTTCTC 3'
F3	2nd	60	S 5' GACATCCCGCGCATCAGC 3'