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Caracterización electrofisiológica de receptores hP2X1 y hP2X1*del* coexpresados en ovocitos de *Xenopus laevis*

Tesis que presenta

Karen Sarahí Gómez Coronado

Para obtener el grado de

Maestra en Ciencias en Biología Molecular

Codirectores del trabajo de tesis:
Dr. Carlos Barajas López
Dra. Alma Rosa Barajas Espinosa

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Constancia de aprobación de la tesis

La tesis ***"Caracterización electrofisiológica de Receptores hP2X1 y hP2X1del coexpresados en ovocitos de Xenopus laevis"*** presentada para obtener el Grado de Maestra en Ciencias en Biología Molecular fue elaborada por **Karen Sarahí Gómez Coronado** y aprobada el doce de diciembre del dos mil dieciocho 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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Dedicatorias

A mis padres Marcial Gómez Pérez y Ma. Carmen Coronado Torres.

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Resumen

Caracterización electrofisiológica de receptores hP2X1 y hP2X1*del* coexpresados en ovocitos de *Xenopus laevis*

Los leucocitos humanos liberan nucleótidos, incluyendo ATP, que al unirse a receptores purinérgicos, son capaces de desencadenar diferentes procesos celulares, incluyendo la muerte de patógenos. El estudio de los mecanismos inmunomoduladores de los receptores P2X en procesos inflamatorios podría elucidar el desarrollo de nuevas estrategias terapéuticas. Existen pocos estudios concernientes al receptor P2X1 de humano (hP2X1) y de su variante de splicing (P2X1*del*), previamente reportada como no funcional. Nuestro laboratorio ha encontrado esta variante de splicing en monocitos humanos, por lo que el objetivo de este trabajo fue caracterizar las propiedades farmacológicas del receptor canónico hP2X1 y su variante de splicing P2X1*del*.

En este estudio, se obtuvieron registros de corrientes inducidas por el ATP, utilizando la técnica de voltage-clamp con dos electrodos en ovocitos de *Xenopus laevis* inyectados con ARNm del receptor hP2X1y P2X1*del*. Las concentraciones utilizadas del agonista fueron: 0.1 a 5000 µM de ATP para hP2X1 y 100 a 5000 µM de ATP para P2X1*del*, también se aplicó suramina a concentraciones de 0.1 a 100 µM para hP2X1. Nuestras observaciones muestran que las corrientes mediadas por el receptor hP2X1 tienen dos fases como respuesta al ATP y que ambas son independientes, ya que una se correlaciona con la apertura de un canal y la otra es más compatible con la apertura de un estado de poro.

Nuestras observaciones indican que uno de los dos sitios de unión tiene alta sensibilidad al ATP y media la activación rápida de una corriente cuya amplitud decrece en pocos segundos (desensibilización), a pesar de la presencia continua del agonista. El segundo sitio de unión tiene baja sensibilidad por ATP, y su estimulación desenlaza una segunda fase en la cual la corriente de entrada aumenta lentamente y su magnitud depende del tiempo de exposición al ATP.

A diferencia de lo reportado, nuestros resultados sugieren que la suramina ejerce dos efectos. El primero es inhibitorio y es evidente a concentraciones altas de suramina (µM), lo que concuerda con lo descrito en la literatura. También descubrimos un novedoso y segundo efecto de potenciación, quizá a través de la estimulación de un segundo sitio de unión con mayor afinidad a la suramina ya que persiste incluso después de su lavado.

Palabras clave: *Receptores P2X1, P2X1del, ATP, Suramina, Voltage- clamp.*

Abstract

Caracterización electrofisiológica de receptores hP2X1 y hP2X1*del* coexpresados en ovocitos de *Xenopus laevis*

Human leukocytes release nucleotides, including ATP, bind to purinergic receptors to trigger a number of cellular process, such as the death of pathogens. The understanding of the immunomodulator mechanisms of nucleotides could help to develop new therapeutic strategies for immune disease and inflammation. Currently, there are few studies on the human P2X1 receptor (hP2X1) and its splicing variant (hP2X1*del*), previously reported as non-functional. Our research group was the first to describe this splicing variant in human monocytes and because little is known about the pharmacological properties of hP2X1 and its splicing variant hP2X1*del*, the aim here was to characterize these properties.

In this study, we measured the currents induced by ATP using the two-electrodes voltaje clamp technique, in oocytes of *Xenopus laevis* injected with hP2X1y P2X1*del* *m*RNA. ATP concentrations ranged from 0.1 to 5000 µM for hP2X1 and 100 to 5000 µM of ATP for hP2X1*del*, whereas 0.1 to 100 µM suramin concentrations were used for hP2X1. Suramin was not tested for currents mediated via the hP2X1*del* receptor. Our results demonstrate that currents mediated by the hP2X1 receptors have two phases and that their activation and inactivation kinetics are independent of each other.

One of the two binding sites has high sensitivity to ATP and mediates the rapid inward current, which decreased in only few seconds, despite the continuous presence of the agonist. The second binding site has a low sensitivity to ATP, causing a second phase, whose amplitude increases slowly as a function of ATP exposure time.

We hereby confirm a suramin elicited effect,that mediates inhibition and is elicited by high concentrations of suramin. However, in contrast to what has been reported, our results suggest that suramin has a second, novel effect at low suramin concentrations, and it likely act via a second binding site with high affinity for suramin producing potentiation rather than inhibition. This effect persists post washing, suggesting that suramin remains attached to the receptor for various minutes.

Key words: *P2X, P2X1del, ATP, Suramin, Voltage- clamp.*

INTRODUCTION

The main role of the immune system is to protect the body against infections and xenogens, which involves its ability to distinguish cells and molecules from foreign bodies. For the immune system to work properly, a fine physiological regulation of organs, cells, proteins, and signaling molecules is required. Several types of blood cells are involved in the immune response and are activated upon contact with a microorganism or particle not recognized by the host (Dantzer et al., 2003).

Nucleotide receptors (P2) in the immune system can act as signals for cellular stress. A response to cellular stress can be manifested in the release of a substance by damaged or dying cells. This response is perceived as a sign of stress and is able to stimulate and induce the differentiation of other cells to mediate a response to ensuing tissue damage. Therefore, extracellular nucleotides, such as ATP, have been considered stress signaling molecules in the immune system (Fig. 1) (Burnstock, 2016; Di Virgilio et al., 2003).

ATP is recognized by purinoceptors (P2X and P2Y) expressed in various cells of the immune system that participate in: cell death, release of prostaglandins, proinflammatory cytokines, regulation of the immune response, activation of transcription factors, cell proliferation, differentiation, and platelet aggregation amongst others (Burnstock, 2001; De Torre-Minguela et al., 2016; Karmakar et al., 2016; Lister et al., 2017).

ATP and Purinergic Receptors

ATP (adenosine triphosphate) is a nucleotide and the main energy source in the cell. ATP is involved in numerous intracellular functions, including synthesis of DNA and RNA, preservation of the cell structure and energy provision for the active transport of macromolecules against their concentration gradient (Goldberg and Waxman, 1985).

Burnstock (2006) described that ATP intervenes in the transmission of intracellular signals but that it also functions as an extracellular transmitter when it is recognized by P2 receptors.

P2X Purinergic Receptors

Purinergic receptors modulate the levels of calcium and cAMP (cyclic adenosine monophosphate) and are activated by extracellular ATP released from cells receiving stress stimuli, electrical impulses, chemical or physical stimuli. ATP is released by different routes, which include: ion channels, specific transporters, vesicles, or by cell lysis. There are two classes of receptors which are activated by adenosine and ATP: the P1 and P2, respectively. In turn, the P2 receptors are divided into two groups, the metabotropic P2Y, coupled to G proteins and the ionotropic P2X receptors (Surprenant and North, 2009).

P2X receptors are membrane proteins, which form ion channels when activated. There are seven subunits, numbered one to seven (P2X1-7), that compose the P2X family.

A functional receptor is formed by three subunits, which is the functional and stable form of these ion channels. The trimers may be of the same subunit (homotrimers)

or different subunits (heterotrimers) (Newbolt et al., 1998). Each subunit is formed by two transmembrane domains, an intracellular domain with an amino and carboxyl terminal group and an extracellular domain (Fig. 2).

Desensitization

When P2X receptors are exposed to ATP, a measurable current is obtained with electrophysiological techniques. This response decreases in the continuous presence of ATP, an effect known as desensitization. P2X1 receptor desensitizes rapidly, as the activated current decreases within few seconds of agonist application (Fig. 3). The recovery of such desensitization requires a prolonged time (3-5 minutes) of stimuli removal. Therefore, subsequent stimulations with ATP induced currents with a lower amplitude than that obtained with the first application. Taking into account these observations, it is suggested that desensitization is mediated by a conformational change in the transmembrane domains, which would inactivate the channel, even in the presence of the agonist (North, 2002).

Physiology and Distribution

ATP has been linked to the activation of inflammatory processes as it act on the P2X7 receptor, which provokes the release of the pro-inflammatory cytokine IL-1β by monocytes and macrophages (Wewers and Sarkar, 2009).

An expression profile of mRNA in human monocytes revealed the expression of subunits P2X4>P2X7>P2X1, in order of monocyte population expression (Wang et al, 2004). However, López et al. (2016) showed that the expression of the P2X1 messenger was more frequently detected (in 90% of the cells) while the expression

of the messenger of the P2X7 subunit was the least expressed (3%) amongst the monocyte population. They also reported the presence of a splicing variant of the P2X1 receptor of human monocytes (*P2X1del*) in 88% of the monocytes. This variant lacks part of exon 6 and codes for a protein that does not possess the 17 amino acids related with the ATP binding site. López et al. (2016) demonstrated this variant to be functional, although Greco et al. (2001) reported this P2X1 splicing variant of human platelets (P2X1*del*) as a non-functional protein.

GENERAL PURPOSE

To characterize the functional and pharmacological properties of the human P2X1 receptor and its splicing variant (P2X1*del*).

SPECIFICS AIMS

To perform the heterologous expression of monocyte hP2X1 sequence and its splicing variant in *Xenopus laevis* oocytes by mRNA microinjection.

To carry out ATP concentration-response curves for both hP2X1 and hP2X1*del*, and concentration-response curve to suramin for hP2X1 using the two-electrode voltage clamp technique.

METHODS

Transformation of competent *E. coli* TOP10 cells

For bacterial transformation, 1.5 µL of cloning canonical receptor hP2X1 in pCDNA3 vector were added to 50 µl of calcium competent *E. coli* TOP10 cells. Cells were transformed by heath shock protocol. Cells were recovered by adding 300 µL of LB medium (2%, tryptone, 0.5% yeast extract, 20 mM glucose, 10 mM NaCl) and incubated at 37 °C for 2 h under continuous agitation at 250 rpm. Bacteria pellet were recovered by centrifugation at 9,500 rpm for 5 min, the supernatant discarded and the pellet was resuspended in the remaining LB medium. Subsequently, 100 µL of the bacteria suspension was seeded on LB-agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar and ampicillin 100 µg/mL) and incubated at 37 °C for 16 h.

Extraction of plasmid DNA for alkaline lysis (midi prep)

Transformed *E. coli* cells were used to inoculate as eppendorf tubes of 25 mL of LB-broth containing ampicillin and incubated for 16 h, at 37 °C and 250 rpm.

Subsequently, the contents of the tube were centrifuged at 6000 rpm for 15 min at 4 °C. The pellet was resuspended with 4 mL Buffer 1 (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mg/ mL RNase A). To perform the lysis, 4 mL of Buffer 2 (1 M NaOH, 20% SDS) was added to the cells, mixed vigorously and incubated for 5 min at room temperature. The sample was neutralized with 4 mL of Buffer 3 (5 M potassium acetate, glacial acetic acid), vigorously shaken and kept on ice for 20 min. Tubes were centrifuged for 30 min at 6000 rpm at 4 °C. The supernatant containing

the plasmid DNA was removed into a fresh tube and 4 mL of isopropanol were added and the reaction was incubated at -20 °C for 20 min and centrifuged at 6000 rpm. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was resuspended with 100 µl of dH_2O. The DNA concentration was determined in the NanoDrop ™ 2000 equipment, using a wavelength of 260 and 280 nm.

Synthesis of mRNA *In vitro*

For *in vitro* mRNA synthesis, 15 µL of the cDNA (hP2X1in pCDNA3 vector) was used as a template and linearized with *Xba I* restriction enzyme (Biolabs™) and the reaction was incubated at 37 °C overnight, after 0.5 µL proteinase K (10 mg / mL) and 1.25 µL of 20% SDS were added to the reaction mix. Finally, the reaction was adjusted to a volume of 100 µl with nuclease-free water and incubated at 50 °C for 15 min. The DNA was purified by adding 1 volume of the mixture phenol:chloroform:isoamyl alcohol (25:24:1); the mixture was mixed by vortex and centrifuged at 13,000 rpm for 3 min. The aqueous phase was recovered and 3 M sodium acetate was added in a ratio of 1:0.1 (v/v) and left on ice for 3 min. Sample was centrifuged at 13,000 rpm for 15 min at 4 °C, the pellet was washed with 1 mL of 70% ethanol and again centrifuged, the pellet was allowed to dry and resuspended in 15 µL of nuclease-free water. The DNA concentration was determined at a wavelength of 260 and 280 nm in a NanoDrop ™ 2000 device. Linearized and purified DNA (1µg) was used to synthesize the mRNA using the mMessage mMachine T7 ultra kit (Life Technologies, Texas, USA), the reaction was left in a thermal cycler at 37 °C for 2 h, then incubated 15 min at 37 °C with TURBO DNAse,

the reagents were added to continue with the last part of mRNA synthesis and left for 45 min at 37 °C in the thermal cycler. The reaction was precipitated with 1/2 volume of LiCl and left on ice for 30 min. It was centrifuged at 13,500 rpm for 15 min at 4 °C and the supernatant was removed. The RNA pellet was washed with 70% ethanol and centrifuged at 13,500 rpm for 5 min at 4 °C. The dry mRNA was dissolved in 15 µL of nuclease-free water and quantified in the NanoDrop ™ 2000. Finally, aliquots of 2 µL were made and stored at -70 °C until required for the microinjection.

Extraction of oocytes from *Xenopus leavis* and mRNA microinjection

A female adult frog of the species *Xenopus laevis* was selected for the extraction of the oocytes. The frog was anesthetized with ethyl methanesulfonate 3-Aminobenzoate salt (Sigma-Aldrich) and the oocytes were removed by dissection. The IV-V phase oocytes were manually removed from the follicle and placed in Barth's solution (88 mM NaCl, 0.33 mM Ca $(NO_3)_2$, 1 mM KCl, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$. and 10 mM HEPES, pH was adjusted to 7.2-7.4 with 5 M NaOH). The oocytes were left overnight at 14 °C to recover before mRNA injection.

mRNA was denatured by incubating at a temperature of 70 °C for 4 min; subsequently, it was kept on ice for 3 min. The micropipettes were made with 3 ½ glass capillaries (Drummond, Scientific Company, USA) in the Flaming / Brown P-87 pipettor puller. The micropipette was filled with mineral oil and placed in the Nanoliter 2000 microinjector (World Precision Instruments). A total volume of 2 µl of

mRNA was aspirated and the oocytes were injected with 36 nL of mRNA, in the clear pole, and incubated at 14 °C for 12-24 h before the electrophysiological experiments.

Electrophysiological assay using the technique of *Voltage Clamp*

Two-Electrode Voltage Clamp (TEVC) recordings were made, using an Axoclamp 2B amplifier (Molecular Devices, Foster City, CA), connected to a MiniDigi 1A analog to digital converter and the AxoScope 9.2 program (Molecular Devices, Foster City, CA). The micropipettes were made with filament capillaries of a resistance between 0.3-0.8 Ω, using the Flaming / Brown P-87 pipette puller and filled with internal solution (2 M KCl, 10 mM EGTA, pH 7.8).

The oocytes were placed in a concave chamber and anchored with the two electrodes in the clear pole using a micromanipulator (WR-88, Narishigue Scientific Instrument Lab, Tokyo Japan). During recordings, the oocytes were continuously superfused with external solution (88 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES pH 7.4) at a rate of approximately 3 mL / min.

The current (I_{ATP}) of each oocyte were normalized considering the response to 100 μM ATP for hP2X1 and the response to 5 mM ATP for hP2X1*del* as 100%.

Solutions stocks of the agonists and antagonists were prepared at a concentration of 100 mM for ATP and 10 mM for suramin (Sigma-Aldrich). Application of the solutions was carried out by means of a “fast” perfusion system with 8 tubes, where a tube with external solution was placed to wash out the drugs.

For drug applications concentrations in the following range were utilized: 0.1 μM to 5000 μM for ATP; 0.1 μM to 100 μM for suramin; and for coapplications 10 μM (ATP), and 0.1 μM to 100 μM (suramin). After drug application, 3 to 5 min washes were

made with external solution to remove the substance(s) prior to the second application. Currents were analyzed with the AxoScope 10.6 software (Molecular Devices, Foster City, CA).

RESULTS

Low concentrations of ATP (0.1- 300 μM) activate the canonical receptor hP2X1

To characterize the hP2X1 mediated currents in *Xenopus laevis* oocytes, the voltage was held at -60 mV and ATP was usually applied for about 10 s (Fig. 4 B). Between each application the oocyte was washed with external solution for 3 min. The hP2X1 receptor showed high sensitivity at low concentrations of ATP and rapid desensitization, a three min wash is sufficient to obtain the initial response amplitude.

High concentrations of ATP induce a second current in the canonical receptor hP2X1

Figure 5 shows the currents induced by high concentrations of ATP (3000 μM and 5000 μM) applied for about 1 min (Fig. 5), with 3 min washes of the oocyte between each application. Both concentrations used evoked two types of currents, the first, was a transient current that rapidly desensitize and a second one was a current that slowly increased with the time of ATP exposure.

P2X1*del* receptor requires concentrations ≥ 3000 μM to induce current.

To characterize the splicing variant hP2X1*del*, ATP concentrations of 300 μM, 1000 μM, 3000 μM and 5000 μM were applied for 30 s, a wash of 3 min was applied between each application. Currents obtained at 3000 μM and 5000 μM ATP increased as the time of exposure to ATP increased. This effect was similar to that observed in the second phase of the hP2X1 (Fig. 5).

Suramin has a dual effect on currents mediated by hP2X1 canonical receptor

To evaluate the effect of suramin, described in the literature as a P2X1 antagonist, concentrations between 0.1 and 100 μM. A control current was recorded using a 10 μM ATP concentration and subsequently the same ATP concentration was co-applied with different concentration of suramin, which was pre-applied 3 min before ATP, the oocyte was washed for 5 min with external solution and then response ATP was recorded again. The amplitude of the response to the co-application was potentiated when low concentrations of suramin (0.1 μM, 0.3 μM, 1 μM) were used and currents return to control values when suramin was washed out. Increasing the suramin concentration to 3 μM, 10 μM, 30 μM and 100 μM, inhibited the ATP induced current but when suramin was washed out during 5 min, transitory potentiation of the currents was observed (Fig. 7). It is likely that these effects are due to the existence of two binding sites for suramin in the P2X1h receptors, one, with a high sensitivity to suramin that mediates potentiation and the second one with lower sensitivity that mediates inhibition of this current.

DISCUSSION

Our results showed that currents mediated by the hP2X1 receptors have two phases and that their activation and inactivation kinetics are independent each other. One of the two bindings sites have high sensitivity for the ATP and mediates the rapid inward current, which decreased in a few seconds, despite the continuous presence of agonist. The second binding site has a low sensitivity to ATP, causing a second phase, whose amplitude increases slowly as a function of the exposure time to ATP.

We also found that suramin can have dual effects on P2X1 receptor, mediated by the high sensitive binding site for ATP, obtained potentiation at low concentrations and inhibition at high concentrations of suramin, which indicates the presence of two binding sites for suramin.

Previous observations from our laboratory showed that the currents mediated by native P2X receptors in monocytes, have also two phases when high concentrations of ATP were used (López et al., 2016). Only the first phase was observed with low concentrations of ATP, this response had high sensitivity to ATP and a fast inward currents was observed, which desensitized quickly, this is in agreement with what has been described in the literature for the P2X1 receptor (Wareham, Vial and Wykes, 2009).

At high ATP concentrations, a second current phase became evident, current that did not desensitize and its amplitude increased as a function of ATP perfusion time (López et al., 2016). It is likely, that these monocyte ATP responses were mediated

mainly by P2X1 receptors. This hypothesis could be tested by additional pharmacological characterization of these receptors in oocytes and monocytes.

Likewise, we showed that the splicing variant hP2X1*del* and hP2X1 was activated with high concentrations of ATP and also that there was an increase in the current amplitude as ATP exposure time was prolonged.

The current obtained in the splicing variant was similar to the response of the second phase presented by the canonical receptor hP2X1. López et al. (2016) reported that the splicing variant, exempt of exon 6, codes for the protein that lacking the 17 amino acids that involve the ATP binding site. This leads us to believe that there are two ATP binding sites in the human P2X1 receptor, due to the fact that losing part of the first ATP binding site in the splicing variant only activates the second binding site that mediates the second phase observed.

In this work, we also investigated the role of suramin in the hP2X1 canonical receptor. Since 1988, Dunn and Blakeley demonstrated that suramin functions as an antagonist of P2X receptors, with the exception of the P2X4 and P2X7 subtypes. Our results showed that suramin has a double effect on hP2X1. The first effect was inhibitory and it was observed at high levels of suramin, in agreement with published results (Greco et al., 2001). The second was a potentiation effect and was observed at low suramin concentrations. This leads us to hypothesize that there exists another high affinity binding site for suramin in the hP2X1 receptor. The potentiator suramin effect has not yet been dissected in the literature yet. A dual effect of suramin has previously been shown in myenteric neurons and their native P2X receptors, this was unlikely mediated by the P2X1 subunit (Guerrero-Alba et al., 2010). This would

suggest that a dual effect of suramin might be present in other receptors different than the P2X1.

It is not clear why the potentiatory effect of suramin persisted after it had been washed out when high concentrations of suramin were used. One possibility is that at such concentrations of suramina, the binding site mediating potentiation, becomes saturated and enough drug remains attached to induced potentiation, this hypothesis in agreement with the high affinity of this binding site for suramin, as observed here.

CONCLUSION

The hP2X1 receptor has two ATP binding sites, one with high sensitivity to ATP, with rapid current and rapid desensitization responses, and another binding site with low sensitivity to ATP, causing a second response phase in which the current increases in the presence of ATP and increasing the current with time.

Suramin exerts a dual effect, indicating that there are two suramin binding sites in the canonical receptor hP2X1, the first mediates the inhibitory effect and it is induced by high suramin concentrations. The other binding site shows a high affinity for suramin and its activation results in current potentiation, a novel effect that is hereby described for the first time on P2X1 receptors.

FIGURES

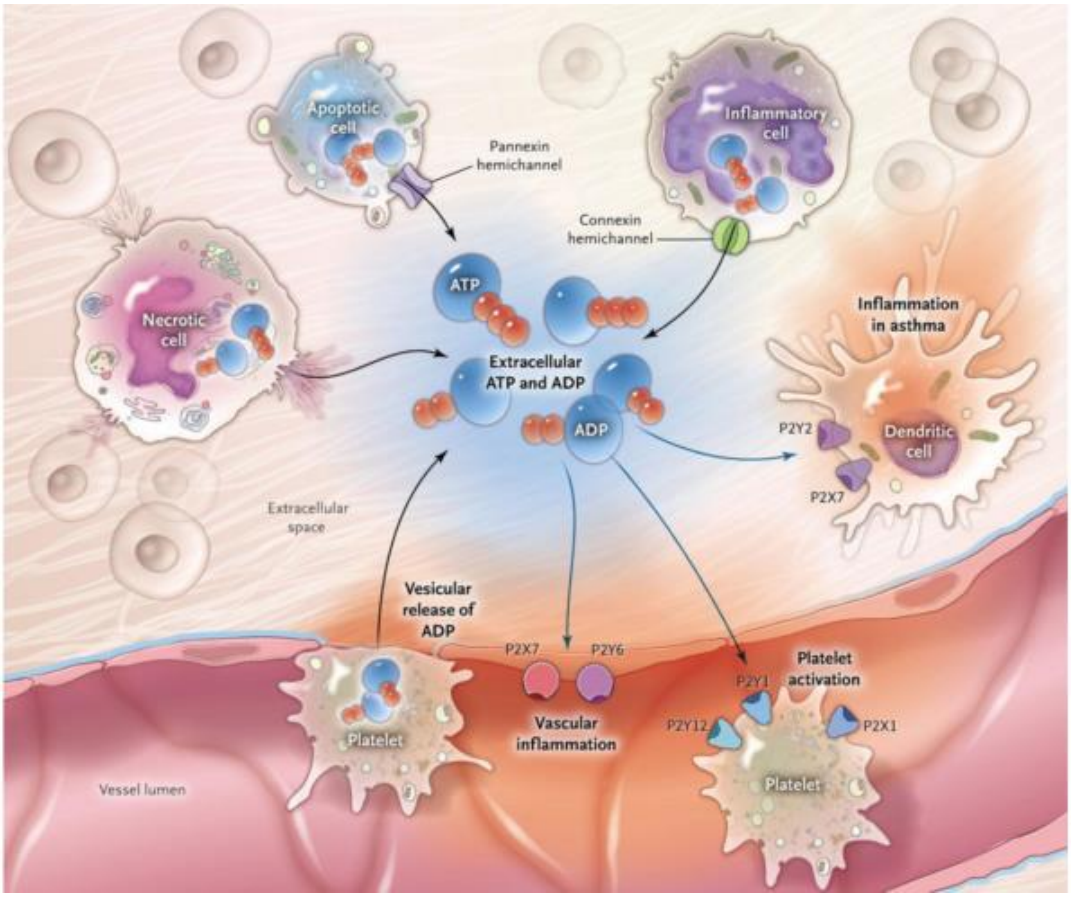


Fig. 1. Nucleotide release and activation of purinoceptors (P2) during inflammation.

During the inflammatory process damaged cells release nucleotides (ATP or ADP) from the intracellular to the extracellular space, which occurs by different mechanisms. For instance, from necrotic cells through hemichannel-dependent pannexins during the apoptosis, through hemichannels of connexin of activated inflammatory cells, by vesicular exocytosis (endothelial cells, astrocytes and activated platelets). In the extracellular space, these nucleotides function as signaling molecules that can activate P2Y receptors (coupled to G proteins) or P2X

receptors (ionotropic channels). Examples of nucleotide receptors signalling under inflammatory conditions include P2Y6 or P2X7 receptors, which mediate vascular inflammation, and P2Y1, P2X1 and P2Y12 receptors that mediate platelet activation (Burnstock G., 2016).

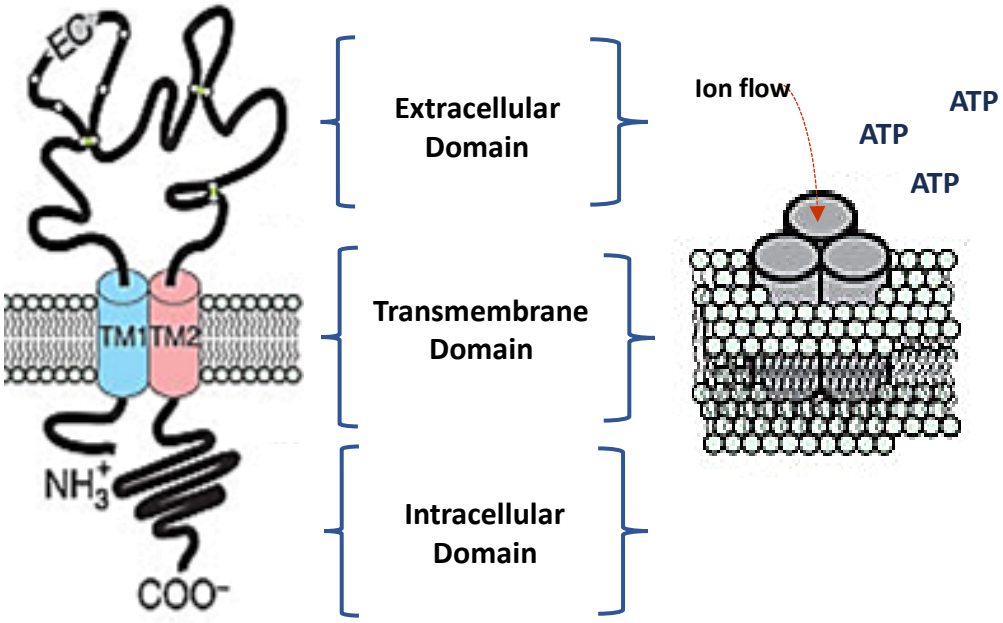
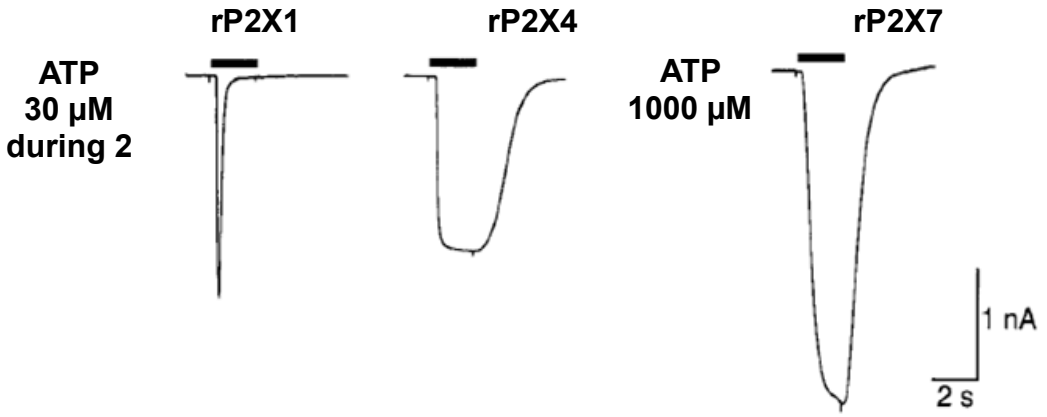


Fig. 2. Structure of the P2X receptor subunits and formation of homotrimers and heterotrimers.

Representation of the structure of subunits to ionotropic receptors of mammals. EC: extracellular domain; TM1 and TM2: transmembrane domains; -COO- and -NH3 +: intracellular domains. Three P2X subunits compose a trimer to form a stable ion channel. These trimers may be of the same subunit (homotrimers) or different subunits (heterotrimers) (Modified from Khakh and Alan North, 2006).



Expression in HEK293 cells.

Fig. 3. Desensitization of rat P2X1, P2X4 and P2X7 receptors.

Rapid desensitization was observed only with P2X1: brief applications (duration of 2 s) of ATP (30 µM for P2X1 and P2X4, and 1 mM for P2X7). Slow desensitization was observed for P2X4 with longer ATP applications (not shown) but not for P2X7 (not shown). HEK293 cells were transfected with 1 µg/mL of cDNA (each in pcDNA3.1) 48 h before these whole-cell recordings were made. The time scale is valid for P2X1 and P2X4 receptors, for P2X7, ATP was applied for 2 min (Modified North, 2002).

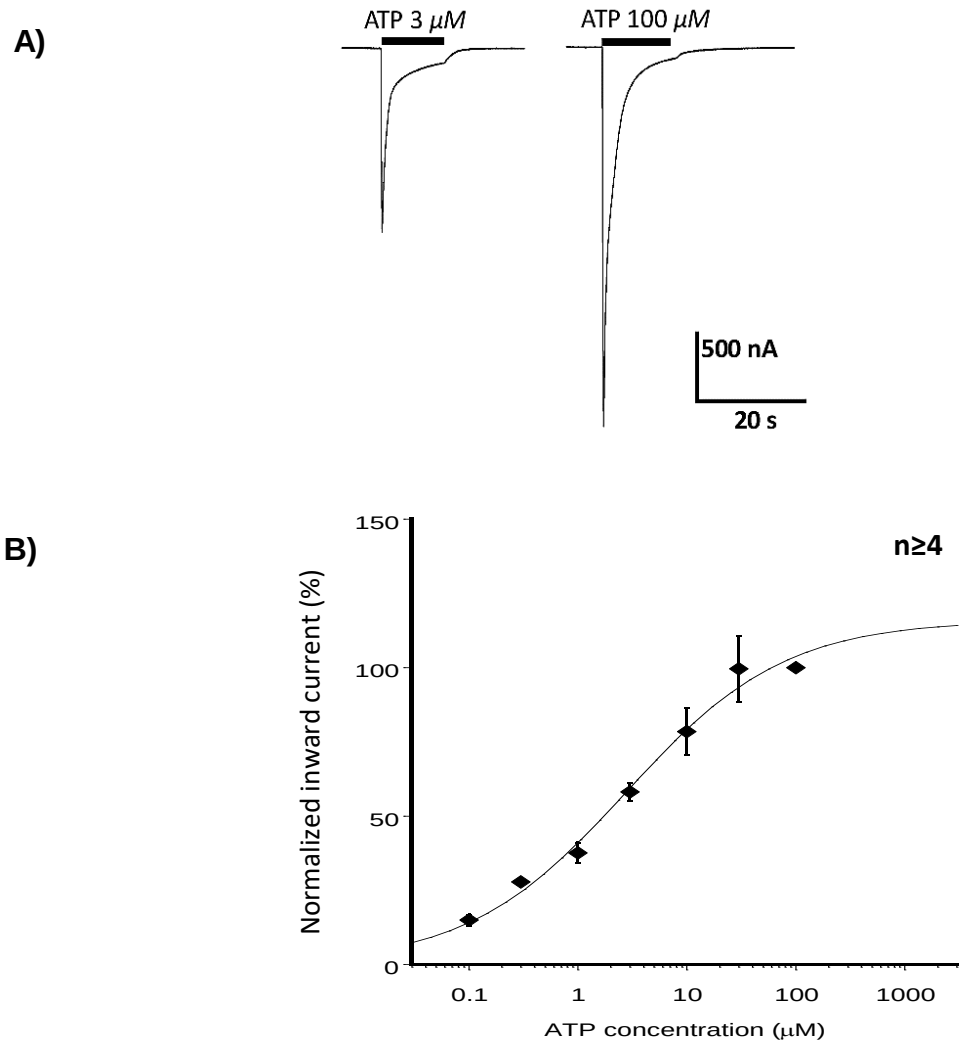


Fig. 4. hP2X1 receptor has a high ATP sensitivity.

(A) ATP-induced currents obtained by applying 3 μM and 100 μM of ATP. Notice the rapid decreased of the current amplitude despite the fact of the continuous presence of ATP. Bars on the top of the currents indicate ATP application. (B) ATP concentration-response curve for receptor hP2X1 (EC_{50}= 2.7 μM) is well fitted with the Hill equation. Symbols represent the current average from 4 to 10 cells. Lines associated with symbols represent the standard error of the mean.

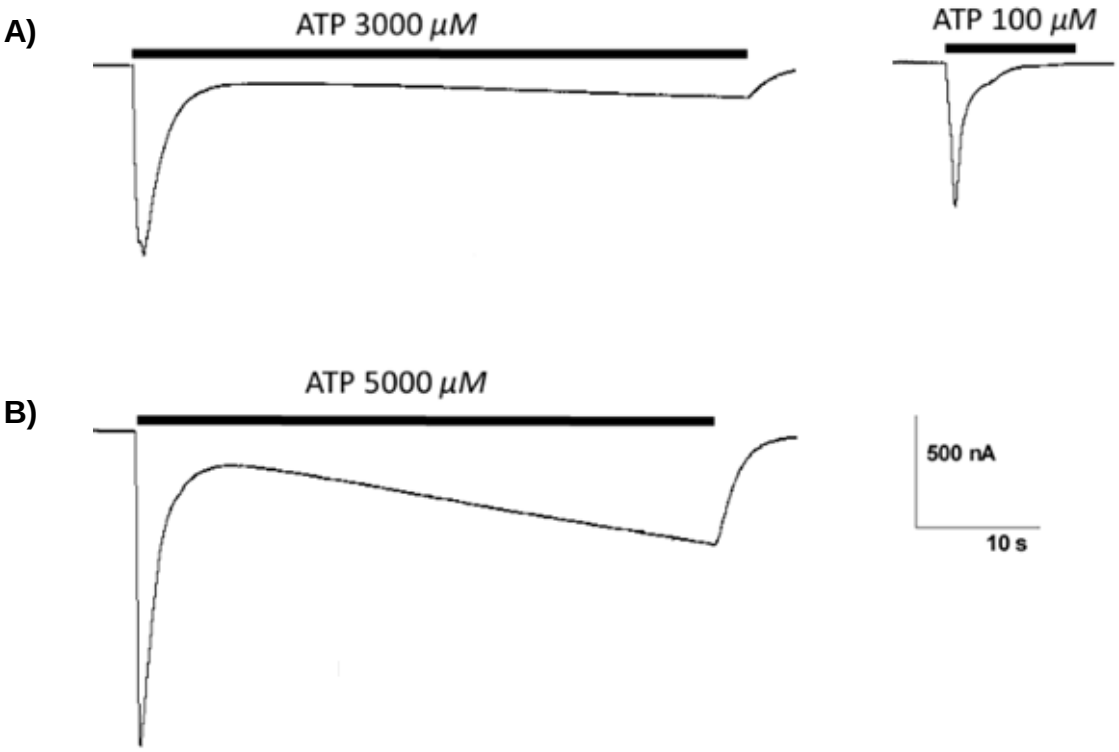


Fig. 5. ATP-induced currents mediated by hP2X1 receptors at high agonist concentrations.

The currents obtained by applying 3000 μM and 5000 μM of ATP are shown. A) and B) show a first response that quickly desensitizes (similar to those currents indicated to the right with 100 μM ATP) and the second response in which the current increases with the agonist time application. The amplitude of this second response, also increased by elevating ATP concentration.

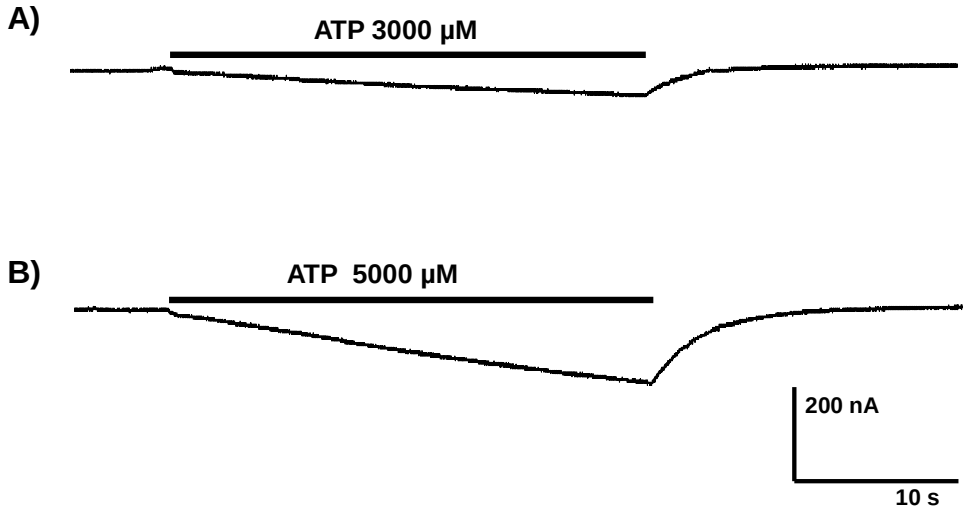


Fig. 6. hP2X1*del* receptors requires ATP concentrations higher than 3000 µM to become activated.

A) and B) the splicing variant hP2X1*del* receptor mediates a current when high concentrations of ATP were applied (3 and 5 mM). This current is similar to the second phase current observed with hP2X1 canonical receptor (Fig. 5).

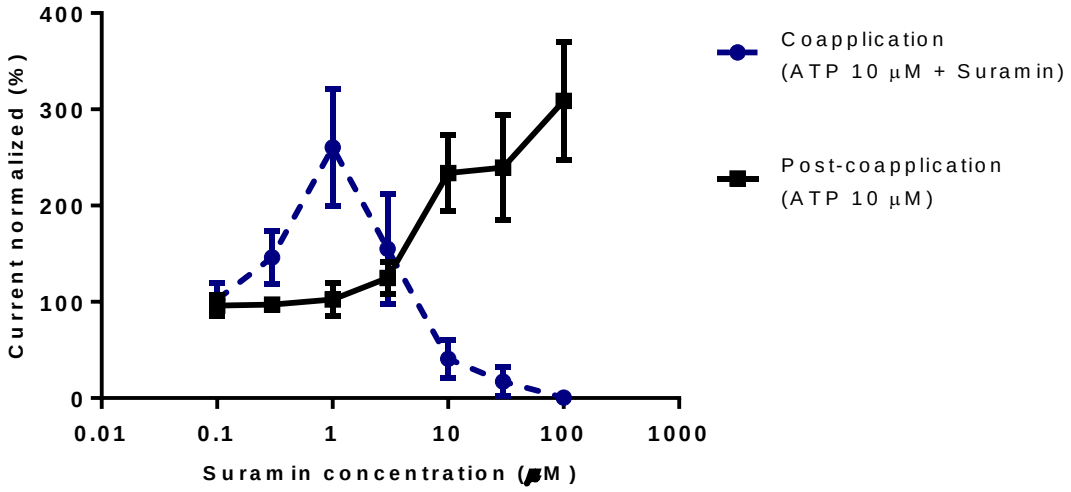


Fig. 7. Suramin has a complex effect on hP2X1 receptor.

The normalized ATP-induced current is showed as a function of suramin concentration suramin, did not induced any current when applied along (not shown). When ATP was applied in the presence of suramin, we observed two effects, at low concentrations (lower than 3 mM), suramin potentiated ATP-induced currents, whereas, at higher concentrations inhibition of the currents were observed. The concentration-response curve of suramin is shown. Concentrations ≤1 μM of suramin (0.1 μM, 0.3 μM and 1 μM) cause a potentiation in the coapplication of suramin with 10 μM ATP (dotted line), and concentrations ≥ 3 μM of suramin (3 μM, 10 μM, 30 μM and 100 μM) produce an inhibition during the coapplication of suramin with 10 μM ATP, but when applying 10 μM ATP (solid line) after a 5 min wash there is a current potentiation. n≥4.

Anexo

Anexo A. Artículos publicados.

López López C., Jaramillo Polanco J., Portales Pérez D.P., Gómez Coronado K.S., Rodríguez Meléndez J.G., Cortés García J.D., Espinosa Luna R., Montaño L.M., Barajas-López C. 2016. **"Two P2X1 receptor transcript sable to form functional channels are present in most human monocytes".** *European Journal of Pharmacology*, vol. *793*: 82–88. doi: 10.1016/j.ejphar.2016.10.033.

DOI: https://doi.org/10.1016/j.ejphar.2016.10.033.

Méndez Barredo L.H., Rodríguez Meléndez J.G., Gómez Coronado K.S., Guerrero Alba R., Valdez Morales E.E., Espinosa Luna R., Barajas Espinosa A., Barajas López C. 2018. **"Physiologycal Concentrations of Zinc Have Dual Effects on P2X Myenteric Receptors of Guinea Pig".** *Cellular and Molecular Neurobiology.* Vol. 38: 1439-1449. doi: 10.1007/s10571-018-0612-7.

DOI: https://doi.org/10.1007/s10571-018-0612-7.

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