Mouse Leydig cells express multiple P2X receptor subunits

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Abstract ATP acts on cellular membranes by interacting with P2X (ionotropic) and P2Y (metabotropic) receptors. Seven homomeric P2X receptors (P2X₁–P2X₇) and seven heteromeric receptors (P2X_{1/2}, P2X_{1/4}, P2X_{1/5}, P2X_{2/3}, P2X_{2/6}, P2X_{4/6}, P2X_{4/7}) have been described. ATP treatment of Leydig cells leads to an increase in [Ca²⁺]_i and testosterone secretion, supporting the hypothesis that Ca²⁺ signaling through purinergic receptors contributes to the process of testosterone secretion in these cells. Mouse Leydig cells have P2X receptors with a pharmacological and biophysical profile resembling P2X₂. In this work, we describe the presence of several P2X receptor subunits in mouse Leydig cells. Western blot experiments showed the presence of P2X₂, P2X₄, P2X₆, and P2X₇ subunits. These results were confirmed by immunofluorescence. Functional results support the hypothesis that heteromeric receptors are present in these cells since 0.5 μM ivermectin induced an increase (131.2±5.9%) and 3 μM ivermectin a decrease (64.2±4.8%) in the whole-cell currents evoked by ATP. These results indicate the presence of functional P2X₄ subunits. P2X₇ receptors were also present, but they were non-functional under the present conditions because dye uptake experiments with Lucifer yellow and ethidium

bromide were negative. We conclude that a heteromeric channel, possibly P2X_{2/4/6}, is present in Leydig cells, but with an electrophysiological and pharmacological phenotype characteristic of the P2X₂ subunit.

Keywords P2X receptors · ATP · Leydig cells · Western blot · Immunofluorescence · Electrophysiology

Introduction

Several studies have shown that extracellular ATP acts as a modulator of function in different endocrine cell types, including Sertoli cells [16], spermatogonia [32], granulosa-luteal cells [47], and adrenocortical fasciculate cells [39]. In Leydig cells of rats and mice, ATP leads to an increase in intracellular calcium concentration ([Ca²⁺]_i) and subsequent testosterone secretion [17, 40]. This effect is due to a calcium modulation of the functioning of the steroidogenic acute regulatory protein (StAR), which represents a limiting step of the steroidogenic process [34, 35]. Although production and secretion of testosterone are controlled mainly by the luteinizing hormone (LH) [37], the above observation supports the hypothesis that ATP is a positive modulator of the process of testosterone production and secretion in Leydig cells.

ATP acts on cellular membranes by interacting with purinergic P2X (ligand-gated ion channels) and P2Y (G protein-coupled) receptors. Seven P2X receptor subunits (P2X_{1–7}) were cloned and characterized in vertebrates, and each of them can form two type of assemblies, homomeric, P2X₁ [50], P2X₂ [3], P2X₃ [8], P2X₄ [2], P2X₅ [18], P2X₇ [46], and P2X₆ [23], or heterotrimeric P2X_{2/3} [31], P2X_{1/5} [48], P2X_{4/6} [30], P2X_{2/6} [27], P2X_{1/2} [5], P2X_{1/4} [38], and P2X_{4/7} [19]. Each subunit is composed by two transmem-

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brane regions (TM1 and TM2) forming the channel pore, a large extracellular domain, which contains the ATP binding site and both N and C termini located intracellularly [14, 48, 22].

Each purinergic receptor assembly presents a particular functional profile [31, 27]. The pharmacological and biophysical properties of homomeric P2X receptors, characterized in heterologous expression systems, allowed their classification into three distinct groups: (1) $\alpha\beta$ -methylene-ATP ($\alpha\beta$ meATP) sensitive and rapidly desensitizing (P2X₁ and P2X₃); (2) $\alpha\beta$ meATP insensitive and slowly desensitizing (P2X₂, P2X₄, P2X₅); and (3) $\alpha\beta$ meATP insensitive, slowly desensitizing and pore formation after long-time exposure to the agonist (P2X₇) [15, 33]. On the other side, P2X₆ receptors form homotrimeric assemblies only after an extensive glycosylation process. When expressed, they show $\alpha\beta$ meATP sensitivity and are slowly desensitizing [23].

Heteromeric assemblies, in most cases, show properties more similar to a given P2X subunit present in the functional receptor, but not exactly equal to the homomeric receptor. Moreover, P2X subunits may have several isoforms, as a result of alternative splicing, which can form heteromeric channels with the same P2X subunits. Each new receptor thus formed has distinct properties when compared with homomeric assemblies [49, 9]. Until now splice variants have been described for P2X₁, P2X₂, P2X₄, P2X₅, and P2X₇ purinergic receptor subunits [20, 9, 12, 13, 7].

Functional purinergic receptors are present in Leydig cells, as demonstrated through electrophysiological and pharmacological assays, suggesting the presence of a phenotype typical of P2X₂ receptors [41]. In fact, the presence of purinergic receptors in Leydig cells was also proposed by Foresta et al. [17] and Perez-Armendariz et al. [40] based on measurements of $[Ca^{2+}]_i$, and also by the finding of P2X₄ and P2X₇ mRNAs [29]. Despite all these studies and the clear implication of purinergic receptors in Leydig cell function, there remains an open question as to the identity of the purinergic receptor subunits present in these cells.

In this paper, we investigated this issue by analyzing both molecular and electrophysiological properties of the receptors. Western blot and immunofluorescence results indicate the presence of P2X₂, P2X₄, P2X₆, and P2X₇ subunits, with distinct spatial distribution. The electrophysiological phenotype of the currents evoked by ATP and the effect of ivermectin on them indicate the presence of heteromers consisting of P2X_{2/4/6}, resembling the properties of homomeric P2X₂ subtype. From a physiological point of view the P2X receptors present in Leydig cells may serve as modulators of the production and secretion of testosterone, by allowing calcium influx into the cell.

Materials and methods

All protocols used in this study were approved by the Institutional Ethical Committee on Animal Experimentation of the School of Medicine of Ribeirão Preto—University of São Paulo (#030/2005).

Leydig cells

Freshly isolated Leydig cells were obtained from 50-day-old Swiss mice (~45 g in weight), after cervical dislocation. The testes were rapidly removed, cleared of their tunica, and placed in Hank's Balanced Salt Solution (HBSS) (Table 1). Cells were isolated following a basic protocol described by Kawa [24] and Carnio and Varanda [6] as follows: each decapsulated testis was carefully infused several times with HBSS with the aid of a syringe and a 25×7-gauge needle. Because Leydig cells are loosely placed in the interstitium between the seminiferous tubules, they were washed away and enter into suspension. For the electrophysiological experiments, they were seeded onto glass coverslips (3 mm×3 mm) and transferred to an experimental chamber continuously perfused with external solution. Leydig cells readily adhere to glass and contaminant cells, mainly erythrocytes, are washed away by the solution flow. Under the microscope, they are easily recognized by their size, round shape, and presence of lipid droplets in the cytoplasm.

Osmotic shock

This procedure was used when a cleaner preparation and a small number of Leydig cells were needed, as in the immunofluorescence experiments. Since most of the contaminating cells are red cells, which are easily disrupted in hypotonic solution, we decided to treat the cell suspension, obtained as described above, with a hyposmotic shock. To this end, the cell suspension was initially centrifuged at 800×g for 3 min and resuspended in a known volume of deionized water for 20 s. After that, a given volume of 10× concentrated HBSS was added to bring osmolality back to control conditions. Cells were centrifuged again at 900×g for 5 min, resuspended and seeded on coverslips. The efficiency of this process was confirmed by cytochemical detection of the enzyme 3β-HSD as described elsewhere [28].

Western blots

The expression of purinergic receptors P2X_{1–7} in Leydig cells was detected by Western blot analysis. Since a large number of high purity Leydig cells are needed for these experiments, we used 20 testes in each preparation. After

Table 1 Bath (HBSS) and pipette solutions (mM)

	NaCl	KCl	CaCl ₂	MgCl ₂	HEPES	D-Glucose	EGTA	TEA	NaHCO ₃	pH ^a
HBSS	140	4.6	1.6	1.13	10	10			5	7.4
Pipette ^b		140		2	10		11	2		7.2

^a pH adjusted with NaOH (bath) or KOH (pipette)

^b Osmolality=308 mosm/kg H₂O (Mark 3 Osmometer—Fiske Associates, Norwood, Massachusetts, USA)

decapsulation, they were treated for 10 min with 0.4 mg/ml collagenase (Type II; Worthington Biochemical Corporation, Lakewood, NJ, USA) and the tubules and cells dispersed by several cycles of suction and ejection with the aid of a Pasteur pipette. Afterwards, Leydig cells were purified from the suspension by centrifugation in a discontinuous Percoll gradient, as described by Schumacher et al. [44]. After purification, they were suspended in radioimmunoprecipitation assay (RIPA) buffer (750 mM NaCl, 250 mM Tris pH 7.5, 5% NP-40, 2.5% sodium deoxycholate, 0.5% SDS, 0.5% Triton X-100) containing a cocktail of protease and phosphatase inhibitors (Sigma Fast Protease Inhibitor), diluted 1:100, respectively. After that, cells were sonicated on an ice bath for 30 s, centrifuged at 10,000×g for 5 min at 4°C and cell lysates collected from the supernatant fractions. Protein concentration was determined using a Bradford Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Each sample of 100 µg of extracted protein was separated by SDS-PAGE 12%. Proteins were then transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) by Bio-Rad Trans-Blot SD semidry transfer cell (Bio-Rad Laboratories Inc., Richmond, CA, USA). Membranes were blocked with 5% BSA in TBST (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h, and then incubated for 1 h with anti-P2X₁₋₇ (1:400) polyclonal antibodies (Alomone Labs Ltd., Jerusalem, Israel) diluted in 5% BSA in TBST. After washing, membranes were incubated for 1 h with goat anti-rabbit IgG conjugated to horseradish peroxidase, diluted in TBST. Finally, the immune complexes were developed with an enhanced chemiluminescence method (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the membranes were then immediately exposed to autoradiographic film (Hyperfilm, Amersham Bioscience, UK).

Immunofluorescence

Leydig cells, isolated by mechanical dispersion and cleaned by the osmotic shock described above, were seeded on coverslips (13 mm diameter) pre-treated with 2% Biobond (Biobond-Tissue Adhesive, Electron Microscopy Sciences, Hatfield, PA, USA), fixed in 4% formaldehyde in 0.01 M PBS (tablets; Sigma Chemical Co., St. Louis, MO, USA) for 15 min and blocked with 0.01 M PBS, 0.5% Triton X-

100, 1% BSA and 5% goat serum for 60 min. The coverslips were incubated 120 min with the same primary antibodies anti-P2X₁₋₇ (1:200; rabbit) and anti-calreticulin (Chemicon International, Temecula, California, USA; 1:200; chicken) diluted with 0.01 M PBS, 0.5% Triton X-100, 1% BSA. Then, the cells were incubated with secondary antibodies Alexa Fluor 488 (goat, anti-rabbit) and 594 (goat, anti-chicken; Molecular Probes Inc., Eugene, USA) for 60 min, both in a dilution of 1:800. All incubations were done at room temperature and cells were washed three times in PBS for 5 min before each incubation. Finally, cells were washed with deionized water and mounted onto glass slides with Prolong (Invitrogen Corporation, Carlsbad, CA, USA). Immunofluorescence analysis was made with the aid of a confocal microscope (TCS SP5, Leica, Bensheim, Germany). The images were analyzed using the software Image J (W. S. Rasband/National Institutes of Health, Bethesda, MD, USA).

Dye uptake

Leydig cells were isolated by mechanical dispersion, purified by osmotic shock and seeded on Petri dishes. ATP application is known to cause permeabilization in cells that express P2X₇ purinergic receptors. To confirm that P2X₇ receptors are functional in Leydig cells, uptake of the fluorescent dyes Lucifer yellow (5 mM; Sigma Chemical Co., St. Louis, MO, USA) and ethidium bromide (10 µM; USB Corporation, Cleveland, OH, USA) was tested by incubating cells in the absence or presence of 5 mM ATP diluted in HBSS. Following a 10-min incubation at room temperature, cells were visualized in the confocal microscope. Cells incubated with Lucifer yellow were washed twice with HBSS before visualization.

Electrophysiological recordings

For electrophysiological experiments, Leydig cells were isolated by simple mechanical dispersion and seeded on glass coverslips (3 mm×3 mm). Whole-cell voltage-clamp recording was carried out at room temperature (23–25°C) using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Membrane potential was held at −50 mV. Recording electrodes (resistance 2–4 MΩ) were

filled with pipette solution (Table 1) and had resistance around 3 M Ω . Access resistance (9.9 ± 0.26 M Ω , $n=218$) and cell capacitance (22.07 ± 0.51 pF) were electronically corrected using the amplifier settings (mean $\pm$ SEM). The bath solution was HBSS. Data were acquired using pCLAMP10 software (Molecular Devices, Sunnyvale, CA, USA). Signals were filtered at 2 kHz, and then digitized at 5 kHz (Digidata 1440A, Molecular Devices, Sunnyvale, CA, USA). Traces were analyzed using Clampfit 10 and/or Origin 7 (Microcal, Northampton, MA, USA). Solutions were delivered by gravity from independent reservoirs using a RSC160 rapid solution changer (Biologic Science Instruments, Claix, France). One barrel was used to apply drug-free solution to enable rapid termination of drug applications. ATP was separately applied for 6 s at 3 min intervals, a washing time sufficient for the responses to be reproducible. Ivermectin was present for 3 min before and during the reapplication of ATP (after one control application). After application of ATP with ivermectin cells were washed for 5 min to remove the drug.

Drugs

Triton X-100 is from Beckman Instruments (Fullerton, CA, USA), ATP, ivermectin, and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Solutions of ATP and other drugs were prepared using deionized water and stored frozen, except for ivermectin, which was dissolved in dimethylsulfoxide (DMSO) to 10 mM. The final concentration of DMSO in the bathing solutions was always <0.01%. All drugs were diluted in the extracellular solution to the desired final concentration.

Data analysis

All whole-cell recordings were normalized to the current evoked by 100 μ M ATP in the same cell. Data are expressed as mean $\pm$ SEM and statistical significance was tested by the Wilcoxon signed rank test using R (version 2.5.1, 2007-06-27, Copyright (C), The R Foundation for Statistical Computing, ISBN 3-900051-07-0).

Results

Partial purification of Leydig cells by osmotic shock

In order to check if the osmotic shock procedure leads to enrichment of the preparation in Leydig cells we performed cytochemical detection of the enzyme 3β -HSD. As can be seen in Fig. 1a and b, the preparation clearly contains Leydig cells (marked in deep blue), but a number of other

cells, mainly red blood cells and spermatozoa, are also present. After the osmotic shock (Fig. 1c and d), much of the contaminating cells disappear, making Leydig cells more evident. This protocol was used thereafter for cell isolation in the immunofluorescence experiments, where a small number of cells is required.

Molecular identification of P2X receptors in mouse Leydig cells

Western Blot experiments were performed in order to detect P2X receptor protein expression in lysates of Leydig cells using subunit specific antibodies against P2X_{1–7} receptors. As shown in Fig. 2a, bands were marked at a molecular weight around 66 kDa, corresponding to the glycosylated form of the P2X₂, P2X₄, and P2X₆ subunits. As far as P2X₇ is concerned, more than one band was marked (Fig. 2b). The band around 75 kDa corresponds to the usual marker for the glycosylated P2X₇ receptor. This is supported by the fact that the same band is also disclosed in lysates of macrophages, used as positive control. Nevertheless, smaller molecular weight bands (around 45 and 53 kDa) are also present, probably representing truncated splice variants of P2X₇. Alternatively, Leydig P2X₇ may have undergone partial degradation. In all cases, the bands are specific for the subunits described above, since pre-absorption of the antibodies with the corresponding cognate peptides knocked down their expression. Figure 2c shows that subunits P2X₁, P2X₃, and P2X₅ are not detected in Leydig cells.

The Western Blot results described above were corroborated by immunofluorescence experiments. Figure 3 shows immunofluorescence labeling due to P2X₂, P2X₄, P2X₆, and P2X₇ receptor subunits (a2, b2, c2, and d2, respectively). Figure 3e2 is a negative control where the primary antibody was omitted during the incubation, indicating specificity of the antibodies used. The fluorescence exhibited by P2X₁, P2X₃, and P2X₅ do not differ from the control experiment. These receptors are absent in Leydig cells corroborating the Western Blot results.

The cellular distribution of P2X₂, P2X₄, P2X₆, and P2X₇ subunits is particular for each type of receptor as shown in Fig. 4 (a1, b1, c1, and d1, respectively). Colocalization with the endoplasmic reticulum (ER) was detected by staining calreticulin and with the nucleus by staining with DAPI. The Manders Overlap Coefficient (MOC) was used as an index of colocalization and calculated using Image J. As can be seen in the merged images (Fig. 4a4 and a5), P2X₂ subunits colocalize with the ER (MOC for P2X₂-ER=0.699) and with the nucleus (MOC P2X₂-NC=0.660). They also form clusters as indicated by the arrow heads. Figure 4b4 and b5 show the merged images for the P2X₄ subunit with respect to

Fig. 1 Cytochemical detection of 3β -HSD on cell suspensions before and after hyposmotic shock. **a** and **b** The cell suspension was obtained by mechanical dispersion only and stained for 3β -HSD. **c** and **d** Mechanically isolated cells were also subjected to a hyposmotic shock and then stained for 3β -HSD. Leydig cells are marked in intense blue. Scale bar 20 μ m

the ER (MOC for $P2X_4$ -ER=0.927) and nucleus (MOC for $P2X_4$ -CN=0.750), respectively. The $P2X_6$ receptor subunit shows the most clustered distribution with MOC for $P2X_6$ -ER=0.816 (Fig. 4c4) and MOC for $P2X_6$ -CN=0.778 (Fig. 4c5). $P2X_7$ subunits also colocalize with the ER (MOC for $P2X_7$ -ER=0.818) and less strongly with the nucleus (MOC $P2X_7$ -NC=0.607). Figure 4d4 and d5 show the merged images for the $P2X_7$ subunit with respect to the ER and nucleus. This indicates that most of these subunits colocalizing with the ER are not functional.

Functional profile of P2X receptors in mouse Leydig cells

To elucidate which receptor subunits are functional in Leydig cells we performed two types of experiments: fluorescent dye uptake for testing the $P2X_7$ receptor and the effect of ivermectin on the ATP-activated currents to look at $P2X_2$, $P2X_4$, and $P2X_6$ receptors.

Figure 5 shows that treatment of Leydig cells with 5 mM ATP for 10 min did not induce any uptake of either Lucifer yellow (Fig. 5a1, a2, and a3) or ethidium bromide

Fig. 2 Western Blot analysis of purinergic receptors $P2X_{1-7}$. **a** Western blot analysis of lysates of mouse Leydig cells performed with specific antibodies against rat $P2X_2$ (lane 1), $P2X_4$ (lane 2), and $P2X_6$ (lane 3) receptors. Positively marked bands are seen at ~66 kDa corresponding to these subunits. These same bands are absent in the experiments where the antibodies were pre-incubated with their respective cognate peptides (lane 1', lane 2', and lane 3' for $P2X_2$, $P2X_4$, and $P2X_6$, respectively). **b** $P2X_7$ receptors were recognized in both Leydig cells (lane 1) and macrophages (lane 2). Again, the

reaction is specific because pre-incubation of the antibody with the cognate peptide resulted in negative results (lane 1' and lane 2', respectively). As expected there are bands marked around 70 kDa, for both Leydig cells and macrophages used as positive control. Besides this, there are three other bands in Leydig cells between 35 and 66 kDa. **c** Antibodies against $P2X_1$, $P2X_3$, and $P2X_5$ receptors did not show any positively marked band. Numbers in the left side of the blots are molecular weight markers

Fig. 3 Immunofluorescence detection of purinergic receptors. Differential interference contrast (DIC) microphotography of Leydig cells (*a1*, *b1*, *c1*, and *d1*) and respective immunofluorescence labeling of P2X₂ (*a2*), P2X₄ (*b2*), P2X₆ (*c2*), and P2X₇ (*d2*) subunits. *e1* is a DIC

image of cells and *e2* the respective immunofluorescence labeling of control experiment, performed in the absence of primary antibodies (*e2*). Scale bar 20 μ m

(Fig. 5b1, b2, and b3) in intact cells, as indicated by the arrows in Fig. 5. There is only a small fluorescent staining in damaged cells. Therefore, although present in Leydig cells, P2X₇ receptors are not functional under the present conditions.

As shown in Fig. 6a, application of 100 μ M ATP to the Leydig cells induces an inward current (at -50 mV holding potential) which slowly desensitizes, as already described [41]. Washing the preparation for 3 min restores the response to control level (Fig. 6a and b, left panels). On the other hand, 0.5 μ M ivermectin causes an increase ($131.2 \pm 5.9\%$; mean $\pm$ SE, $n=12$, $p<0.05$) in the ATP elicited current (Fig. 6a and b, middle panels) while 3 μ M ivermectin leads to a significant reduction in the ATP current ($64.2 \pm 4.8\%$ mean $\pm$ SE, $n=19$, $p<0.05$, Fig. 6a and b, right panels). These results indicate that the phenotype of the currents evoked by ATP can be determined by heteromers containing P2X₄ subunits.

Taken together our results indicate the presence of multiple receptor subtypes in Leydig cells. Our main finding is that the receptor may be formed by a heteromeric assembly of P2X₂, P2X₄, and P2X₆ subunits with an electrophysiological and pharmacological phenotype mainly determined by the P2X₂ subunit.

Discussion

Leydig cells are easily isolated using a mechanical dispersion method. However, for immunofluorescence experiments it is easier to localize these cells when the

preparation is made free of other cell types. To achieve a preparation with lower contamination, we associated mechanical dispersion with a hyposmotic shock. This new procedure results in a less-contaminated Leydig cell preparation. This conclusion is confirmed by the results shown on Fig. 1, where the cells were recognized by cytochemical detection of the enzyme 3 β -HSD.

Purinergic receptors in mouse Leydig cells show fast activation and slow desensitization, mostly resembling the properties of homomeric P2X₂ ionotropic receptors [41]. However, the Western Blot results show the presence of bands for four different subtypes of purinergic receptors, P2X₂, P2X₄, P2X₆, and P2X₇ (Fig. 2a, lanes 1, 2, and 3; b, lane 1; respectively). We also find three other bands marked by the anti-P2X₇ antibody, with lower molecular weights. In this experiment, positive control with macrophage cells, which have endogenous expression of P2X₇ homomeric receptors [11], confirms the band corresponding to the P2X₇ subunit at approximately 70 kDa. Therefore, Leydig cells endogenously express the classical P2X₇ subunits and other P2X₇ subunits probably derived from alternative splice. It is known that expression of alternatively spliced subunits does not always result in functional channels, almost certainly because of lacking of protein segments important for their function [12, 7]. Seven isoforms of alternatively spliced human P2X₇ purinergic receptors were already described. For mice P2X₇ receptors, four isoforms were described, “a”, “b”, “c”, and “d” (NCBI respective numbers: NP 035157, NP 001033934, NP 001033928, and NP 001033976). The larger isoform “a” is 99% similar to

Fig. 4 Immunofluorescence detection of purinergic receptors. Immunofluorescence cellular localization of P2X₂ (a1), P2X₄ (b1), P2X₆ (c1) and P2X₇ (d1) subunits. Colocalization of purinergic receptors and endoplasmatic reticulum (ER) by Immunofluorescent staining of ER and respective merge with P2X₂ (a2; a4), P2X₄ (b2; b4), P2X₆ (c2; c4), and P2X₇ (d2; d4) subunits. Colocalization of purinergic receptors and cell nucleus (CN) by immunofluorescent staining of CN

and respective merge with P2X₂ (a3; a5), P2X₄ (b3; b5), P2X₆ (c3; c5), and P2X₇ (d3; d5) subunits. *White arrow heads* indicate clusters of P2X subunits. P2X₂ colocalized with ER and CN and P2X₆ with the nucleus. *White color* on merged images indicates colocalization points. P2X₇ is the only subunit that do not colocalize with CN. *Scale bar* 10 μ m

mice P2X₇ subunit and the other three are not recognized by antibody used for Western Blot experiments.

The immunofluorescence experiments corroborated the Western Blot results, showing staining for anti-P2X₂, anti-P2X₄, anti-P2X₆, and anti-P2X₇ antibodies (Fig. 3, a2, b2, c2, and d2, respectively). Each subunit shows a distinct distribution and colocalization with the endoplasmatic reticulum (ER) and the cell Nucleus (CN). Staining of the P2X₂ subunit reveals a sparse distribution and appears as agglomerates of fluorescent points colocalized with both ER and CN (Fig. 4a). The P2X₆ subunit agglomerates are mainly colocalized with the CN (Fig. 4c). The agglomerated points observed for both P2X₂ and P2X₆ subunits represent “clusters” of more than three equal purinergic subunits, which do not form functional channels. These

clusters stay in the ER and are not incorporated into the plasma membrane [1]. This point is particularly relevant for the P2X₆ subunits, which requires an extensive glycosylation process in order to form functional homotrimeric channels [23]. The P2X₄ subunit is colocalized with the ER and CN, showing a dense and uniform distribution (Fig. 4b4 and b5). The P2X₇ subunit appears as stained points distributed mainly close to the membrane and also colocalized with the ER (Fig. 4d4 and d5). Since all subunits show colocalization with the ER and, in this compartment they are non-functional, we can assume that this may represent a modulatory step for the receptors expression in the plasma membrane.

Despite the fact that purinergic receptors present in Leydig cells show functional properties consistent with

Fig. 5 Fluorescent dye uptake by Leydig cells. DIC image of cells (*a1*, *b1*), and respective visualization of Lucifer yellow fluorescence in cells before and after incubation with 5 mM ATP for 10 min (*a2*, *b2*, respectively), and merge (*a3*, *b3*). DIC image (*c1*, *d1*) and respective ethidium bromide fluorescence in cells before and after incubation with 5 mM ATP for 10 min (*c2*, *d2*, respectively), and merge (*c3*, *d3*). Intact cells do not present fluorescent staining for both dyes (*white arrows*) after incubation with ATP. Scale bar 20 μ m

homomeric P2X₂ receptors [41], our present results suggest that P2X₂, P2X₄, P2X₆, and P2X₇ subunits can interact to form heteromeric channels. To elucidate the assembly of functional purinergic receptors, we analyzed other functional properties of these receptors. P2X₇ homomeric receptors show singular properties in comparison to other purinergic subunits. Pharmacologically, the main agonist for native mice receptors is not ATP (EC_{50} =734 μ M), like other subunits, but the analog BzATP (EC_{50} =90 μ M) [10]. Leydig cells show an EC_{50} for ATP around 44 μ M [41], which are not consistent with the presence of homomeric P2X₇ receptors. Moreover, the necessity of millimolar concentra-

tions of ATP to stimulate these channels *in vivo* poses a question about the possible physiological role of these receptors [21]. Another singular property of these receptors is that they have a large pore-forming phenotype specific for P2X₇ after long-term stimulation with the agonist, allowing large molecules (>900 Da) to pass [46]. Our results on dye uptake in intact Leydig cells are negative for both Lucifer yellow (457.24 Da; Fig. 5a and b) and ethidium bromide (394.31 Da; Fig. 5c and d). Only damaged cells show some fluorescence for both dyes. These results are consistent with the absence of functional P2X₇ receptors in Leydig cells under our experimental conditions.

Fig. 6 Effect of ivermectin on ATP-activated currents. **a** Representative recordings of inward currents evoked by sequential applications of 100 μ M ATP for 6 s. The holding potential was -50 mV in all cases. The *left panel* shows control traces in response to three applications of ATP (ATP1, ATP2, and ATP3), indicating that desensitization of the receptor is removed under these conditions. The *middle panel* refers to the application of ATP in the presence of 0.5 μ M ivermectin (ATP2). There is a clear increase in the current amplitude in relation to control (ATP1) which is reversed upon wash out of IVM. The *right panel* shows ATP-evoked current before (ATP1) and after incubation with

3 μ M IVM. A small decrease in the amplitude of the ATP-evoked current (ATP2) is now observed, which is partially reversed upon wash out of the drug (ATP3). **b** Bar graphs of the peak current amplitudes evoked by applications of ATP in control conditions (*left panel*, $n=17$ cells), after incubation with 0.5 μ M IVM (*middle panel*, $n=10$ cells), and after incubation with 3 μ M IVM (*right panel*, $n=11$ cells). Numbers are normalized to the first control condition taken as 100%. Bars represent mean $\pm$ SEM (* $p < 0.05$, Wilcoxon test). Washout interval between second and third applications was 3 and 5 min, respectively

Ivermectin is known to act only on P2X₄ receptors by binding to two distinct sites. The higher-affinity binding site ($EC_{50}=0.25$ μ M) is able to increase the maximal ATP-activated currents without changing the ATP concentration–response relationship. The action at the lower affinity binding site ($EC_{50}=2$ μ M) results in a slower deactivation rate and enhancement of the ATP affinity [43]. Moreover, ivermectin does not act on P2X₂, P2X₃, P2X_{2/3}, and P2X₇ receptors but does so on heteromeric channels containing the P2X₄ subunit [25, 4, 19]. After ATP stimulation of Leydig cells, we observed a 30% non-reversible increased in the peak currents subsequent to the incubation with 0.5 μ M ivermectin (Fig. 6a and b, middle panel). However, a reversible decrease of the peak currents, amounting to 35%, was detected by using 3 μ M ivermectin (Fig. 6a and b, right panel). The increase in current supports the idea that P2X₄ subunit is part of the receptor. Furthermore, the current decrease, which was not expected if only P2X₄ subunits were present, indicates that other subunits are involved in the receptor assembly [43]. Conversely, if P2X_{4/7} heteromeric receptors were present, we should have observed an increase on the peak currents after 3 min of incubation with 3 μ M ivermectin, a fact not seen in our results [4, 19]. Heteromeric P2X_{4/6} receptors show high affinity for $\alpha\beta$ MeATP (threshold=10 μ M) [25]. Neverthe-

less, purinergic receptors in mouse Leydig cells do not present purinergic currents after stimulation with 100 μ M $\alpha\beta$ MeATP [41]. Therefore, we can exclude the possibility of functional P2X_{4/6} heteromeric receptors in Leydig cells.

The presence of homomeric P2X₂ receptors can also be detected by observing the response to ATP at different extracellular pHs. P2X₂ homomeric receptors show only an increase in peak currents when pH is changed from control (pH 7.4) to a pH of 6.5 [26, 45, 51]. In addition, pH variations to 5.5 caused an increase in P2X₂ peak currents but a decrease on P2X_{2/6} peak currents [27]. In mouse Leydig cells, bringing the pH to 5.5 induced a decrease on peak currents [41], corroborating the hypothesis that functional purinergic receptors are formed by P2X₂ and P2X₆ subunits. In summary, our results suggest that purinergic receptors in mouse Leydig cells are heteromers of P2X_{2/4/6} subunits, although showing a dominant functional profile of P2X₂ subunits [41]. Similar results were seen on Purkinje neurons of postnatal rats [36]. We also show that P2X₇ subunits, probably alternatively spliced variants, are present in mouse Leydig cells but certainly non-functional.

From a physiological perspective, it should be noted that LH leads to testosterone secretion in Leydig cells by increasing the intracellular concentrations of cAMP and

calcium. Although the levels of intracellular calcium ions can be controlled in other ways, Foresta et al. [17] and Perez-Armendariz et al. [40] have shown that ATP induces an increase in testosterone production and secretion coupled to an increase in the intracellular calcium concentration. These results support the hypothesis that ATP may play a modulatory role, either autocrine and/or paracrine, on this process. In keeping with this hypothesis, our laboratory has recently shown the presence of volume-activated chloride channels in Leydig cells, which may serve as a pathway for ATP release [42].

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