P2X receptors in mouse Leydig cells

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P2X receptors are a family of ionotropic purinergic receptors that are expressed in a wide range of cells and tissues. They are activated by ATP and consist of several subtypes, each with distinct biophysical and pharmacological properties. Therefore, the identification of the subtypes present in native cells is an important step in understanding the physiological roles of these receptors. Because selective antagonists are not readily available for the P2X receptors, a collection of properties derived from studies in heterologous expression systems has been used to infer the composition of P2X receptors in native cells.

The recombinant homomeric P2X1 and P2X3 receptors are readily distinguishable by their rapid desensitization and response to αβ-methylene-ATP (αβ-MeATP) (6, 43), whereas recombinant homomeric P2X2 (4) and P2X5 (16) desensitize slowly and are not activated by αβ-MeATP. The recombinant homomeric P2X7 desensitizes slowly and is almost insensitive to the nonspecific antagonists suramin and PPADS (46). Although recombinant P2X7 receptors show pronounced differences among species, it is worth noting that the mouse P2X4 receptor is in fact potentiated by suramin, by reactive blue 2, and, depending on the concentration, also by PPADS (41). The recombinant P2X6, which desensitizes slowly, is blocked by suramin and PPADS (8), but is poorly expressed in heterologous systems and probably does not form functional homomeric channels in vivo (40). The P2X7, which does not desensitize, has 2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP) as the more potent agonist, and its activation is followed by formation of large membrane pores (37). Although P2X channels were described as essentially selective to cations, being highly permeable to Ca2+, recombinant P2X5 receptors are Cl− permeable (2, 32). Furthermore, in response to long applications of ATP, the recombinant P2X5 and P2X4 channels can change their selectivity, allowing the passage of large cations such as N-methyl-D-glucamine (NMDG) (20, 45).

In Leydig cells, production and secretion of testosterone are processes controlled mainly by LH, which binds to surface receptors and initiates a cascade of intracellular events leading to increases in the intracellular levels of cAMP and Ca2+ (36). Several other substances act as modulators of this process, including gonadotropin-releasing hormone (GnRH), growth factors, and cytokines (34).

In recent years, ATP was added to the list of modulators of the steroidogenic process in the testes. Treatment of rat Leydig cells with ATP leads to an increase in testosterone secretion (15) and evokes Ca2+ release from intracellular stores as well as Ca2+ influx from the extracellular medium (30), indicating that ATP signaling may contribute to the control of Leydig cell function. These findings were reinforced by the detection of mRNA for P2X4 (46) and P2X5 (9) in whole testis tissue preparations. Although these studies provided strong evidence for a role of P2X receptors in Leydig cell function, a detailed

ATP HAS BEEN DESCRIBED as a signaling molecule that can be coreleased with neurotransmitters in peripheral and central neurons (13, 14), as an autocrine/paracrine agent released from nonneural cells in response to mechanical perturbations such as membrane stretch (10), as transported through the CFTR protein itself or through an associated protein controlled by CFTR (42), and as released from various cell types by mechanisms that remain poorly understood.

Extracellular ATP binds to two classes of purinergic receptors, the P2X, which are ligand-gated ion channels, and the P2Y metabotropic receptors, which exert their effects via G proteins. To date, seven P2X receptor subunits (P2X1−P2X7) have been cloned from mammalian tissues (29), and some of them undergo alternative splicing (13). P2X subunits combine to form homomeric or heteromultimeric channels (40) with distinct biophysical and pharmacological properties. Therefore, the identification of the subtypes present in native cells is an important step in understanding the physiological roles of these receptors. Because selective antagonists are not readily available for the P2X receptors, a collection of properties derived from studies in heterologous expression systems has been used to infer the composition of P2X receptors in native cells.

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examination of receptor types present in these cells is lacking. In the present study, we addressed this issue by using the patch-clamp technique to analyze biophysical and pharmacological properties of P2X receptors in freshly isolated Leydig cells. Compared with results from other tissues as well as from heterologous expression systems, our data indicate that Leydig cells express functional P2X2 receptors.

MATERIALS AND METHODS

The experimental protocols used in this work were reviewed and approved by the Institutional Ethical Committee for Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo.

**Leydig cell isolation.** Adult male Swiss mice (60–65 days old) were killed by cervical dislocation, and the testes were immediately removed and placed into HBSS containing (in mM) 145 NaCl, 4.6 KCl, 1.6 CaCl₂, 1.2 MgCl₂, 10 d-glucose, 5 NaHCO₃, and 10 HEPES, pH adjusted to 7.4 with NaOH (300–310 mosmol/kg H₂O). A Leydig cell suspension was obtained without enzymatic treatment by carefully washing the interstitium of decapsulated testes with HBSS, as previously described by Kawa (19) and Camio and Varanda (5). The cells were plated onto glass coverslips and allowed to adhere. The main contaminants in our preparations were red blood cells, which were washed out by perfusing the experimental chamber. The isolated Leydig cells were positively identified by cytochemical staining for the presence of the enzyme 3β-hydroxysteroid dehydrogenase (22), a characteristic bright ring that was visible under phase-contrast microscopy, and could easily be distinguished from other cells.

**Electrophysiological recordings.** Cells were transferred to a Lucite chamber (volume 300 μl) mounted on the stage of an inverted microscope (TMD, Nikon, Tokyo, Japan), and continuously perfused at a rate of 1–2 ml/min with HBSS. Recording pipettes were pulled from borosilicate glass capillaries (BF150-86-15, Sutter Instrument, Novato, CA) on a P-97 puller (Sutter Instrument) and had resistances in the range of 3–5 MΩ for whole cell current measurements or 12–15 MΩ for single-channel recordings. The Leydig cells used in the present experiments had an average resting potential of −14.1 ± 0.7 mV and a capacitance of 23.4 ± 0.5 pF (n = 63). Series resistance was 11.7 ± 0.6 MΩ (n = 63) and was electronically corrected by −60–70%. The relatively low resting potential is a characteristic of Leydig cells dialyzed with a Na⁺-free solution (11). Pipettes were filled with an intracellular solution containing (in mM) 150 KCl, 3 CaCl₂, 1 MgCl₂, 10 HEPES, and 5 EGTA, pH adjusted to 7.4 with KOH (290–300 mosmol/kg H₂O; final Ca²⁺ concentration was 10⁻⁶ M). Pipettes were fire polished and coated with Sylgard (Dow Corning, Midland, MI) for the single-channel recordings.

Currents were measured with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA), following standard techniques. They were low-pass filtered at 2 kHz (−3 dB frequency, Bessel filter, 80 dB/decade) and sampled at 1–5 kHz when in the whole cell configuration or sampled at 10 kHz for the single-channel measurements. Cells were held at −60 mV or as indicated in specific experiments. In some whole cell experiments, voltage ramps from −80 to +40 mV were applied (300-ms duration). Voltage protocols and data acquisition were accomplished with the aid of a personal computer equipped with an analog-to-digital/digital-to-analog converter (Digidata 1200, Axon Instruments) controlled using pCLAMP 6.0.4 software (Axon Instruments). The pipette was connected to the amplifier with an Ag/AgCl wire and the bath to ground by an Ag/AgCl wire and an agar bridge (2.5% in the pipette solution). When pH was set to values ≤6.5 or ≥8.0, HEPES was substituted by MES or MOPS, respectively.

For measurement of permeability ratios, pipettes were filled with the following solution (in mM): 170 NaCl, 10 Cl⁻, and 10 EGTA (pH adjusted to 7.4 with NaOH and osmolality 300–310 mosmol/kg H₂O). The difference in the concentrations of Na⁺ and Cl⁻ are due to the addition of NaOH for pH adjustment. A 3 M KCl agar bridge was used as the indifferent electrode. The external solutions used were (in mM) 150 NaCl, 10 HEPES (solution 1); 75 NaCl, 10 HEPES (solution 2); 50 CaCl₂, 75 NaCl, 10 HEPES (solution 3); and 155 NMDG-Cl, 10 HEPES (solution 4). All solutions had osmolality levels in the range 300–310 mosmol/kg H₂O adjusted with glucose and pH 7.4 adjusted with NaOH or HCl in the case of the NMDG solution. Gigaseal and whole cell configuration were first obtained in normal HBSS, and then the bath solution was changed to solutions 1, 2, 3, and 4 for measurement of the respective reversal potentials (Erev) in the absence and presence of ATP. Liquid junction potentials did not exceed 2.3 mV with the solutions used, and no corrections were made. The permeability ratios (Pd/PNa) were derived from Erev as follows (2, 44):

$$P_{SMCG}/P_{Na} = \exp^x$$

$$P_{Cl}/P_{Na} = \left[\frac{1 - \exp(-x)}{[Na_+] - [Na_+]\exp(x)}\right]/\left[[Cl_+]\exp(-x)-[Cl_+]\right]$$

$$P_{Cl}/P_{Na} = \left[\frac{[Na_+]\exp(x)[1 + \exp(x)] + P_{Cl}/P_{Na}[1 + \exp(x)]}{[[Cl_+]\exp(x) - [Cl_+]\exp(4/[Ca_+])}\right]$$

where x = Erev/RT (F, R, and T are Faraday constant, gas constant, and temperature, respectively) and [I] and [I] refer to specific ion activities inside and outside the cell, respectively. We assumed activity coefficients of 0.75 for Na⁺, 0.75 for Cl⁻, and 0.25 for Ca²⁺. Drugs were applied to the cells via a gravity-driven superfusion system through a series of nine glass capillaries (inner diameter ~0.7 mm), each containing a given solution, placed 100–200 μm from the cell surface, at a rate of 70 μl/min and controlled by a system of electrovalves (RSC-160 system, BioLogic Science Instruments, Grenoble, France).

Data analysis: Unless stated otherwise, the current responses were normalized to that evoked by the maximal concentration used in the experiment or the maximal current obtained. Data are presented as means ± SE for the given number of observations (n). Plots of concentration-response curves were fitted using the Hill equation: $I/I_{max} = [ATP]^{H}/(K_A + [ATP]^{H})$, where I is current, $I_{max}$ is the maximal current, $K_A$ is the Hill coefficient, and ATP is ATP concentration. Experimental points in the specific literature of Figs. 3, A and B, and 6D were fitted by a single exponential equation, and those in Fig. 5B were fitted by a logistic biphasic sigmoidal function. Desensitization was measured during 60 s of continuous application of 100 μM ATP. This method was used to evaluate desensitization because the time course of the decay of the current varied considerably among cells, requiring from one to three exponential components for an adequate fit (47). Traces were analyzed and plotted with Clampfit 8.0 (Axon Instruments) and Origin 6.0 software (Microcal, Northampton, MA). The free Ca²⁺ concentrations in the intracellular pipette solution were calculated with MaxQuelator (Chris Patton, Stanford University; http://www.stanford.edu/~cpatton).

Drugs: PPADS was from Tocris Cookson (Bristol, UK); ATP (sodium salt), αβ-MeATP (lithium salt), 2-MeS-ATP, ATPγS, UTP (sodium salt), GTP, adenosine, cAMP, BzATP, suramin, and all other salts were obtained from Sigma (St. Louis, MO). Stock solutions (10–100 mM) of ATP and other drugs were prepared with deionized water and stored frozen until the time of usage. All drugs were diluted in the extracellular bathing solution to the required concentration before application to cells. All experiments were done at room temperature (23 ± 1°C).

RESULTS

**Pharmacological profile of ATP-evoked currents.** Under whole cell voltage clamp (membrane potential ($V_m$) = −60
mV], application of ATP evoked a dose-dependent inward current in Leydig cells (Fig. 1). Figure 1A shows representative traces obtained from a cell exposed to ATP ranging from 1 to 300 μM. Data from this type of experiment were used to construct a concentration-response curve (Fig. 1C). The experimental points were well described by the Hill equation with a $K_d$ of $44.2 \pm 1.8 \mu M$ and a $n_H$ of $2.2 \pm 0.1$ ($n = 5$ cells), suggesting that at least two ATP molecules bind to the receptor. The concentration-response curve did not change with the holding voltage ($-80$ to $+40$ mV; results not shown).

The following set of experiments was done to check the efficacy of different purinergic agonists on eliciting current in Leydig cells. These receptors could be activated by ATP, ATPγS, 2-MeS-ATP, and BzATP, which evoked 88%, 70%, and 14%, respectively, of the current evoked by the same ATP concentration (100 μM) (Fig. 1B, inset). Therefore, the order of efficacy of the agonists was ATP > ATPγS > 2-MeS-ATP > BzATP. No current was generated by αβ-MeATP, GTP, UTP, cAMP, or adenosine (Fig. 1B), even at concentrations $\geq 100$ μM. Concentration-response curves for ATPγS and 2-MeS-ATP yielded $K_d$ values of $110.2 \pm 6.2$ and $637.0 \pm 45.8$ μM and $n_H$ of $1.8 \pm 0.1$ and $1.9 \pm 0.2$, respectively ($n = 5–8$ cells; Fig. 1C).

The currents elicited by ATP in Leydig cells were readily blocked by suramin and PPADS, as shown in Fig. 2. Block by suramin was noncompetitive (Fig. 2A), and 200 μM reduced the current by 75% in relation to that evoked by 300 μM ATP. The effects of suramin were completely reversible. PPADS, on the other hand, had a more persistent effect, which could not be fully reversed even for washout times $\geq 8$ min (Fig. 2B, right). We also tested whether αβ-MeATP could have an antagonist effect. At a concentration of 500 μM, αβ-MeATP did not block the currents evoked by 100 μM ATP (Fig. 2C).

Desensitization and recovery of ATP-elicited currents. The currents elicited by ATP slowly declined with time during a

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Fig. 1. ATP and ATP analogs activated currents in Leydig cells. A: typical recordings of a cell exposed to the indicated concentrations of ATP for 3 s (horizontal bars over each trace). B: inward currents evoked by ATP and various agonists (100 μM, 5-s application). Inset, maximal currents evoked by ATP analogs (■) relative to that evoked by ATP (100 μM) ($I_{ATP}$); bars represent means ± SE ($n = 9$ cells). The washout interval between the applications was 3 min. C: agonist concentration-response for ATP (■), ATPγS (●), and 2-MeSATP (○). Results are plotted as % of maximal current ($I_{max}$) for each agonist; each point is mean ± SE ($n = 3–8$ cells). The continuous line is the best fit of the Hill equation to the experimental points, resulting in a concentration evoking 50% of maximal ATP response ($K_d$) of 44, 110, and 637 μM and a Hill coefficient ($n_H$) of 2.1, 1.8, and 1.9 for ATP, ATPγS, and 2-MeS-ATP, respectively.

Fig. 2. ATP-activated currents are blocked by suramin and PPADS, as shown in Fig. 2. Block by suramin was noncompetitive (Fig. 2A), and 200 μM reduced the current by 75% in relation to that evoked by 300 μM ATP. The effects of suramin were completely reversible. PPADS, on the other hand, had a more persistent effect, which could not be fully reversed even for washout times $\geq 8$ min (Fig. 2B, right). We also tested whether αβ-MeATP could have an antagonist effect. At a concentration of 500 μM, αβ-MeATP did not block the currents evoked by 100 μM ATP (Fig. 2C).
sustained application of the agonist (Fig. 3A, inset). We quantified this desensitization by calculating the ratio of the currents observed after 60 s in the presence of ATP and the value of the peak after the cell was exposed to ATP ($I_{60}/I_{max}$). Desensitization increased with the concentration of ATP as shown in Fig. 3A. At the end of 60 s of ATP application, the current decayed by $12 \pm 0.1\%$, $48 \pm 0.1\%$, $55 \pm 0.1\%$, and $69 \pm 0.1\%$ with 10, 60, 100, and 200 μM ATP ($n = 10$), respectively. Figure 3B shows that recovery from desensitization is also a slow process. The relationship between the percent recovery from a 5-s pulse of 100 μM ATP vs. washout time was well fitted by a single exponential function with a time constant of 41 s, and at least 3 min of washout was required to fully recover the current amplitude observed at the first application of the agonist.

Ion selectivity. Figure 4A shows the current-voltage plot obtained when 100 μM ATP was applied to the cells in normal HBSS. The current reversed at $2.9 \pm 1$ mV ($n = 5$ cells), and the ratio of the currents at $-20$ and $-80$ mV was 0.15, indicating a strong rectification. To evaluate the ionic basis of this current, current-voltage relationships were obtained by applying voltage ramps between $-80$ and $+40$ mV, at the peak
of the current response to ATP application, and permeability ratios in relation to Na\(^+\) were determined by measuring changes in the \(E_{rev}\) for Cl\(^-\), Ca\(^{2+}\), and NMDG\(^+\).

Changing solution 1 for 80 mM Na\(^+\) and 75 mM Cl\(^-\) (\(E_{Na} = -18.9\) mV, \(E_{Cl} = 16.6\) mV) shifted \(E_{rev}\) to \(-18.4 \pm 0.8\) mV (\(n = 9\) cells; Fig. 4B). This indicated that the channels were practically impermeable to Cl\(^-\) (\(P_{Cl}/P_{Na} = 0.03\)) because this value was very close to that predicted by the Nernst equation for Na\(^+\). In the presence of 50 mM Ca\(^{2+}\) (solution 3), \(E_{rev}\) shifted to \(0.8 \pm 1\) mV (\(n = 9\) cells) and the calculated permeability ratio \(P_{Ca}/P_{Na} = 5.32\) (Fig. 4C). \(E_{rev}\) measured when NMDG\(^+\) (155 mM, solution 4) was the only cation in the bathing solution was shifted to the left (\(-61.3 \pm 0.9\) mV; \(n = 3\) cells), resulting in \(P_{NMDG}/P_{Na} = 0.09\) (Fig. 4D).

**pH effects on purinergic receptor-activated currents.** The pH sensitivity of P2X receptors was reported to vary among the different subtypes (35). Therefore, to investigate the pH dependence of the ATP currents, we adjusted the pH of the extracellular solution to 5.0, 5.5, 6.0, 6.5, 7.4, 8.0, and 8.6 and measured the current responses to 100 \(\mu\)M ATP. The results in Fig. 5 showed maximal currents at pH \(-6.6\), which sharply decreased on both alkalination and acidification of the bathing solution.

**Single channels.** In control outside-out patches, no single-channel events were seen, particularly at hyperpolarized potentials. Application of 50 \(\mu\)M ATP to outside-out patches resulted in the appearance of single-channel activity. The single-channel currents were of relatively small magnitude, even at potentials as negative as \(-100\) mV (\(-1.86 \pm 0.23\) pA at \(-100\) mV; \(n = 7\)), with the occurrence of openings at all potentials studied (Fig. 6A). Figure 6B shows an all-points current amplitude distribution for a recording made at \(-20\) mV. This particular voltage was chosen to show that we were able to discriminate channel openings and closings even for such low-magnitude currents. From similar plots obtained at different \(V_m\), we constructed the current-voltage relationship for the open single channels shown in Fig. 6C. The relationship is clearly nonlinear, showing a strong rectification that resembled that seen for the macroscopic current-voltage relationship. This result suggested that rectification is a property of the single channel and may be responsible, at least in part, for the strong rectification seen in the whole cell experiments. In fact, the ratio of the single-channel current observed at \(-20\) mV to that at \(-80\) mV is 0.17, a value very close to that calculated from the whole cell results. We also note that the experimental points could be clearly described by a single exponential function (no model implied here) that crossed the voltage axis at 6.9 mV, when extrapolated to 10 mV, suggesting the same selectivity as that of the whole cell measurements. A linear fit to the points between the voltages \(-50\) and \(-90\) mV resulted in a chord conductance equal to 27 pS.

During continuous application of ATP, the channels tend to open less frequently and eventually disappear after 2–3 min. Reapplication of ATP resulted in new single-channel activity only after extensive washout for minutes, supporting the idea that the system is nonstationary in time. Moreover, we rarely observed a patch having only one channel. Both of these facts make kinetics analysis difficult. To measure open probability as a function of time, we calculated the average current flowing through the patch as a function of time, noting that \(I = i_o NP_o\), where \(I\) is the mean current flowing through the patch in a given time, \(i_o\) is the single-channel current, \(N\) is the number of channels in the patch, and \(P_o\) is the probability of having an open channel. Results from observations in four cells kept at a holding voltage of \(-90\) mV are shown in Fig. 6D. The mean ionic current declined with time (\(\tau = 13\) s), resembling the desensitization behavior seen with the whole cell current (Fig. 3A, inset).

**DISCUSSION**

Several studies have shown that extracellular ATP plays a role in both short- and long-term cellular communication, including excitable and nonexcitable cells. For example, in sympathetic nerve terminals, P2X receptors participate in the neurotransmission process (3), and in pituitary gonadotrophs, they allow Ca\(^{2+}\) influx and hormone secretion (38) and are involved in skeletal muscle development (33) and in the control of proliferation and differentiation of cultured human keratinocytes (17).

The present study was aimed at identifying and characterizing purinoceptors in mouse Leydig cells and was motivated by the following observations: 1) ATP has been shown to induce testosterone secretion in rat Leydig cells (15); 2) ATP has been shown to increase intracellular Ca\(^{2+}\) concentration in rat Leydig cells (15, 30); and 3) the type of receptor present in these

![Fig. 5. pH effect on ATP-evoked currents. A: typical responses of a cell exposed to 100 \(\mu\)M ATP and bathed by external solutions with the indicated pH. Horizontal bars correspond to 10 s. B: currents were normalized to the value measured at pH 7.4, taken as control, and plotted against the pH of the bathing solution. The maximal response was achieved at pH 6.6. The continuous line represents the best fit of a biphasic sigmoidal equation to the experimental points, suggesting 2 apparent acidic dissociation constant (pK\(_a\)) values: 5.9 and 7.4. Points are means \(\pm\) SE; \(n = 15\) cells.](http://ajpcell.physiology.org/)
cells is still obscure, making their functional role difficult to assess. To achieve our goal, we examined the electrophysiological properties of the currents evoked by ATP and the pharmacological profile of the receptor with purinergic agonists and antagonists in freshly isolated mouse Leydig cells.

In general terms, ATP elicited a current with a fast rise time (on the order of milliseconds), dependent on the concentration of the agonist, blocked by both suramin and PPADS, and slowly desensitizing (Figs. 1A, 2, A and B, and 3A, inset). These findings are typical of ligand-gated P2X receptors (29).

**Pharmacology.** The rate of desensitization of the ATP-evoked currents in Leydig cells could be classified as slow compared with the response in other native cells (24, 44) or in expression systems of recombinant homomeric P2X1 and P2X3 (6, 43) and heteromeric P2X1/3 (24), P2X1/5 (39), P2X2/3 (25), and P2X4/6 (23) channels. Additionally, αβ-MeATP was unable to elicit current and did not have any blocking effect on the ATP-evoked current (Figs. 1B and 2C). Because in cells heterologously expressing homomeric P2X1 and P2X3 (6, 43) and heteromeric P2X1/3 (24), P2X1/5 (39), P2X2/3 (25), and P2X4/6 (23) currents could be activated by low concentrations (1–50 μM) of αβ-MeATP, it is unlikely that these receptors are responsible for generating the ATP-activated currents we observed in mouse Leydig cells. On the other hand, the slow desensitization rate and lack of activity of αβ-MeATP in Leydig cells were similar to those seen in cells heterologously expressing homomeric P2X2 (4), P2X3 (16), or P2X7 (37) receptors.

The homomeric P2X4 receptor is almost insensitive to antagonists effective at other P2X receptors such as P2X2 (46). Strikingly, for the mouse P2X4 splice variants, suramin and PPADS actually potentiated the current instead of blocking it, as for other P2X subunits (41). Furthermore, 100 μM αβ-MeATP is able to elicit 29% of the current evoked by the same concentration of ATP in P2X4 receptors (18). Thus the pharmacological profile of the P2X4 receptor seems to be very different from that of the P2X receptor we observed in Leydig cells.

Homomeric P2X7 receptors are characterized by the fact that BzATP is a more effective agonist than ATP (37). In the Leydig cell, ATP was a more potent agonist than BzATP, making it unlikely that P2X7 receptors underlie the currents we reported.

Therefore, by comparing our pharmacological and desensitization results with those of ATP-evoked currents in recombinant P2X receptors expressed in a heterologous system, we can infer that the receptors present in Leydig cells have properties that more closely match those of homomeric P2X2 and/or P2X5.

**Ionic permeability.** The relative permeability to calcium (P_{Ca}/P_{Na} = 5.32) observed for the P2X receptors in Leydig cells is a common characteristic of recombinant P2X receptors studied under bi-ionic conditions (2, 43, 44).

The relative permeability to Cl\(^-\) in recombinant P2X5 ortholog receptors [chicken P_{Cl}/P_{Na} = 0.5 (32); human P_{Cl}/P_{Na} = 0.52 (2)] has been shown to be a common characteristic of this homomeric receptor compared with other P2X receptors. The P2X Leydig receptors were practically impermeable to Cl\(^-\) (P_{Cl}/P_{Na} = 0.03) and had a very small relative permeability to NMDG (P_{NMDG}/P_{Na} = 0.09), suggesting that...
we are not dealing with homomeric P2X$_2$. The NMDG permeability values reported for Leydig cells were the same as those observed for P2X$_2$ (44). In our case, the permeability to NMDG did not change significantly with time (up to 40 s; results not shown), as reported by others (20, 45). This may reflect differences in expression systems and/or the presence of splice variants with distinct characteristics.

**pH effects.** The pH dependence of the currents elicited by ATP is taken as a key feature of P2X$_2$ receptors (7). In fact, North (29) takes the increased response to ATP with acidification as an important property of this type of receptor. King et al. (21) showed, for recombinant P2X$_2$ receptors, that the ATP-induced current tended to increase with acidification of the extracellular solution, with a peak at pH 6.5 (pH was changed from 8.0 to 5.5), and Stoop et al. (35) also showed, for recombinant P2X$_2$ receptors, that the ATP-induced current in the control (pH 7.3) tends to increase with acidification of the extracellular solution (pH 6.3), whereas alkalization to pH 8.3 caused a strong inhibition of the currents. Unlike P2X$_2$ receptors, P2X$_1$, P2X$_3$, and P2X$_4$ receptors decrease their apparent affinity with acidification (35).

In the present study, we also examined the effects of pH on the ATP-induced current in the pH range 8.6 –5.0 and found a biphasic behavior (Fig. 5). The ATP current was maximal at pH 6.5 and decreased for both alkalization and acidification. The changes in current during alkalization could be understood by assuming the presence of positively charged amino acids, such as histidine, in the region responsible for ATP binding, as suggested by Clyne et al. (7). This interpretation was supported by the apparent acidic dissociation constant ($pK_a$) of 7.4 encountered in our experiments (Fig. 5B), similar to the apparent $pK_a$ of 7.05 and 7.3 found by King et al. (21) and Stoop et al. (35), respectively. Therefore, in the alkaline pH range, the reduced amplitude of the response to ATP could be explained by deprotonation of histidine residues and consequent reduced affinity of the ATP for the binding site.

Less explored in the literature is the decrease in current with acidification to pH values <6.5. At these pH values, the reduction in the ATP-induced currents could be explained by assuming that the effective concentration of one or more negatively charged forms of the ATP molecule was decreased. This point of view was supported by the finding of an apparent $pK_a$ of 5.9, compatible with the $pK_a$ of the phosphate group of ATP (6.2–6.4). In fact, King et al. (21) calculated the free ATP$^{4-}$ present in solutions with different pH values and found a clear decrease in its concentration as pH fell.

**Single-channel currents.** The results of single-channel recordings shown here are the first description of unitary conductance of purinergic receptors in Leydig cells. Contrary to reports in other types of native cells (48), our measurements showed quite similar results from cell to cell, indicating the possibility that we were dealing with one population of receptors, formed by either a homomeric channel or a heteromeric channel with the prevalence of a given subunit. Our recordings resembled those of Ding and Sachs (12) made in HEK-293 cells transfected to express P2X$_2$ receptors. Channels had a chord conductance of 30 pS in symmetrical NaCl solution at −100 mV and were blocked by calcium with a dissociation constant of 3.4 mM. Our extracellular solution had 1.6 mM CaCl$_2$; therefore, blocking should not have been intense in our experiments, and the single-channel chord conductance for voltages between −90 and −50 mV was 27 pS. The current-voltage relationship reported by Ding and Sachs (12) was clearly rectifying, as in our case.

For channels measured in PC-12 cells (from which P2X$_2$ receptors were originally cloned), Neuhaus et al. (28) reported a single-channel conductance of 26 pS, which rectified and decreased by raising the extracellular calcium concentration. In hypothalamic paraventricular parvocells, Whittlock et al. (48) reported the presence of a heterogeneous population of channels, with different kinetic properties and current-voltage relationships. Nevertheless, they suggested that P2X$_2$ subunits were present in these cells, because some of the characteristics of the channels were similar to those observed in P2X$_2$ seen in PC-12 cells, as shown by Nakazawa and Hess (27). These last authors reported that the channel strongly rectified and was blocked by external calcium with a $K_d$ equal to 6 mM, and the opening of the channel induced a current −4.3 pA at −150 mV (roughly 30 pS at this voltage). Zhou and Galligan (49) reported conductance of 25 pS, between −60 and −120 mV, with a small rectification in myenteric neurons.

Our results for the single-channel display several of the features pointed out above, mainly, flicker, strong rectification, and unitary conductance (Fig. 6) in the same range. It is also interesting to note that the ratios of the currents at −20 and −80 mV obtained in the microscopic and macroscopic current-voltage relationships, 0.15 and 0.17, respectively, are almost identical, reinforcing the idea that the voltage dependence of the system could be almost entirely accounted for by the rectification at the single-channel level, as also suggested in the paper by Zhou and Hume (50). Besides this, the single-channel activity decays with time, also resembling the macroscopic desensitization.

**Physiological implications.** In pituitary gonadotrophs, purinergic receptors, probably P2X$_2$ and/or P2X$_3$, have been implicated in calcium signaling and hormone release (38). The ATP-induced current shows the same agonist pharmacological profile seen by us in Leydig cells and was not sensitive to αβ-MeATP. Interestingly, ATP is cosecreted with LH on stimulation by GnRH, providing a mechanism for positive feedback, which can be controlled by the ecto-ATPases present in this tissue.

Testosterone secretion in Leydig cells is coupled to an increase in intracellular cAMP and Ca$^{2+}$ concentrations (36). Calcium ions seem to be involved in the translocation of cholesterol to the inner mitochondrial compartment (26). Although not conclusively established, the increase in cytosolic Ca$^{2+}$ concentration is due to both an intracellular releasable pool and Ca$^{2+}$ influx from the extracellular solution (31). Despite these facts, there is only one report describing the presence of Ca$^{2+}$ currents in Leydig cells (19). Therefore, because purinergic receptors of the P2X type are readily permeable to calcium ions, they could serve as a pathway for the entry of calcium. This is in agreement with the findings of Perez-Armendariz et al. (30) and Foresta et al. (15) showing that ATP increased both Ca$^{2+}$ concentration and testosterone secretion in Leydig cells.

Although the functional properties described here are entirely consistent with those observed for P2X$_2$ receptors and are not consistent with those of any other subtype, it should be recognized that a small number of heteromeric receptors or of
other homomeric receptors might be present although domi-
nated by P2X2 behavior.

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