Role of P2-purinergic receptors in rat Leydig cell steroidogenesis

Carlo FORESTA*‡, Marco ROSSATO*, Andrea NOGARA*, Francesco GOTTARDELLO*, Paola BORDON* and Francesco DI VIRGILIO†
*Patologia Medica III, Università di Padova, Via Ospedale 105, 35128 Padova, Italy, and †Institute of General Pathology, University of Ferrara, Ferrara, Italy

The present study investigated the effects of extracellular ATP on the intracellular calcium ion concentration ($[Ca^{2+}]_i$) and testosterone production in isolated adult rat Leydig cells. This nucleotide caused an increase in $[Ca^{2+}]_i$, with a maximal effect at a concentration of 100 μM ATP, comprising a rapid initial spike followed by a long-lasting plateau. The first rapid spike was dependent on the release of Ca^{2+} from internal stores, since it also occurred in Ca^{2+}-free medium and was abolished after depletion of internal stores with thapsigargin. The second, long-lasting, phase was dependent on the influx of Ca^{2+} from the extracellular medium. After 3 h of incubation, extracellular ATP stimulated testosterone secretion in a dose-dependent manner, with a maximal effect at 100 μM. Activation of steroidogenesis by ATP was fully dependent on the presence of Ca^{2+} in the external medium. Among different nucleotides, only ATP, adenosine 5′-[γ-thio]triphosphate, UTP, benzoylbenzoic-ATP and 2-methylthio-ATP were effective in inducing both the rise in $[Ca^{2+}]_i$ and testosterone secretion. These effects were blocked by preincubation of Leydig cells with oxidized ATP, an inhibitor of the P2Z-purinergic receptor subtype. These results show that rat Leydig cells possess P2-purinergic receptors whose activation triggers an increase in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from internal stores and Ca^{2+} influx from the external medium. The stimulatory effect of extracellular ATP on testosterone secretion seems to be coupled to the influx of Ca^{2+} from the external medium.

INTRODUCTION

It is well known that luteinizing hormone is the main physiological regulator of Leydig cell steroidogenesis [1], although several lines of evidence suggest that other peptides (corticotropin-releasing hormone, atrial natriuretic peptide, erythropoietin, luteinizing hormone-releasing hormone) [2–5] and neurotransmitters (5-hydroxytryptamine, dopamine, catecholamines) [6,7] may also participate in the modulation of this process.

A number of studies have shown that extracellular ATP regulates cellular functions in a variety of endocrine cells. This nucleotide stimulates secretion of insulin and glucagon from isolated pancreatic islets [8–10], influences thyroid and pituitary functions *in vitro* [11,12] and induces catecholamine release from rat pheochromocytoma cells [13]. It has also been demonstrated that extracellular ATP, acting at specific P2-purinergic receptors, stimulates steroidogenesis in the adrenocortical fasciculata and in granulosa cells of both animal and human origin [14]. Plasma membrane P2-purinergic receptors are classified according to their pharmacological profiles into distinct subtypes: P2Y, P2U, P2X, P2Z and P2T [15]. Three main signal-transducing mechanisms triggered by activation of these receptors have been identified as: (1) activation of a G-protein-coupled phospholipase C, leading to PtdIns hydrolysis and generation of Ins(1,4,5)P_3 and diacylglycerol; (2) opening of cation (Na^+, Ca^{2+} and K^+) channels; and (3) formation of plasma membrane pores permeable to molecules of low molecular mass (up to 900 Da). These ATP-dependent early responses are followed by a cascade of intracellular processes, including stimulation of Ca^{2+}-dependent enzymes, stimulation of phospholipase A_2 and activation of Ca^{2+}-dependent exocytotic events [15].

Although P2-purinergic receptors have been identified in many different mammalian cell types, the presence of these receptors in Leydig cells and the possible biological action of extracellular ATP in such cells have not been reported. In the present study we have investigated the effects of extracellular ATP on testosterone secretion in isolated adult rat Leydig cells and the possible mechanisms involved.

EXPERIMENTAL

Materials

Medium M-199 containing Hanks salts and L-glutamine, penicillin and streptomycin were obtained from Gibco (Grand Island, NY, U.S.A.); collagenase (type II), BSA (fraction V), Hepes and soybean trypsin inhibitor (type 1s) were from Sigma (St. Louis, MO, U.S.A.); Percoll and Density Marker Beads were from Pharmacia Fine Chemicals AB (Uppsala, Sweden); silicone fluid was from Serva (Heidelberg, Germany). Fura-2/AM was obtained from Molecular Probes (Eugene, OR, U.S.A.). ATP and all other nucleotides were purchased from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of analytical grade.

Isolation and purification of Leydig cells

Adult male rats of the Sprague–Dawley strain (body weight 280–310 g) were used. The animals were housed in a controlled environment (22 °C, with a 14 h-light/10-h dark photoperiod). Food and water were available *ad libitum*. Interstitial cells were prepared from testes by decapsulation and collagenase digestion as previously described [4]. Briefly, 12–14 testes were incubated with M-199 (3 ml/testis) containing Hanks salts and L-glutamine,

Abbreviations used: ATP[S], adenosine 5′-[γ-thio]triphosphate; BzATP, benzoylbenzoic-ATP; 2-MeSATP, 2-methylthio-ATP; oATP, oxidized ATP; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration.

‡ To whom correspondence should be addressed.

plus 0.2 % BSA (fraction V) and 1 g/l collagenase (type II), at 34 °C in a shaking water bath (90 cycles/min) under a controlled atmosphere (95 % O_2/5 % CO_2). After 15–20 min the suspension was filtered through sterile nylon gauze (mesh 0.5–0.8 mm) and erythrocytes were removed (about 75–80 %) by the addition of 5 ml of 60 % (v/v) Percoll to the bottom of each tube, followed by centrifugation at 800 ***g*** for 10 min at 22 °C. After washing twice, the cells were resuspended in M-199, and 5 ml of the interstitial cell suspension [$(20–25) \times 10^6$ cells/ml] was layered on the top of a vial containing a previously prepared discontinuous density gradient of Percoll (0–60 %, v/v), followed by centrifugation at 800 ***g*** for 20 min at room temperature. The fractions were collected from the bottom of the tubes using a peristaltic pump and then washed twice with isotonic M-199 (1:1, v/v) to remove any residual Percoll. The cells were then resuspended in M-199, and Leydig cells (92–95 % staining positively for 3β-hydroxysteroid dehydrogenase activity [16]) were distributed in a band at the density corresponding to 40–55 % Percoll. The cell concentration (1.0×10^6 Leydig cells/ml) and viability (over 90 %) were determined using a haemocytometer and by the Trypan Blue method respectively.

Incubation of purified Leydig cells

Aliquots (0.5 ml) of Leydig cell suspension (1.0×10^6 cells/ml) were incubated in M-199 with Hanks salts and L-glutamine, plus Hepes (10 mM), 0.2 % BSA (fraction V), penicillin (10 units/l), streptomycin (1 g/l), at pH 7.4 with Tris(hydroxymethyl)-aminomethane (1 M), in polyethylene sterile tubes containing ATP (concentration range 1 μM–1 mM), in a shaking water bath (90 cycles/min) at 34 °C under a controlled atmosphere (O_2/CO_2, 19:1). Aliquots (0.5 ml) of Leydig cell suspensions containing ATP were incubated in the presence and absence of extracellular calcium. After 3 h, the incubation was stopped by immersion of all tubes in an ice-cooled water bath, followed immediately by centrifugation at 1500 ***g*** for 15 min at 4 °C. Supernatants were stored at −20 °C until assayed.

Measurement of the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in Leydig cell suspensions

The Leydig cell $[Ca^{2+}]_i$ was measured using the fluorescent probe fura-2/AM. Briefly, cells were suspended in a standard saline containing 95 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM fructose, 25 mM $NaHCO_3$, 1.7 mM $CaCl_2$, 0.25 mM sodium pyruvate, 10^4 units/ml penicillin, 10 mg/ml streptomycin, 20 mM Hepes (pH 7.4 at 37 °C) and 0.3 % BSA. Fura-2/AM (5 μM) was added and the incubation carried out for 45 min at 37 °C. After loading, cells were washed free of extracellular dye by centrifugation (250 ***g*** for 3×10 min at room temperature) in standard saline and then resuspended at a density of 2.5×10^5/ml. Aliquots of 2 ml of this suspension were utilized for $[Ca^{2+}]_i$ determination. Cells were kept at room temperature until used. All experiments were completed within 2 h of fura-2 loading. $[Ca^{2+}]_i$ was determined as previously described [17].

Determination of Mn^{2+} influx

Mn^{2+} uptake was measured by monitoring the rate of fluorescence quenching at the excitation wavelength of 360 nm (isosbestic point). When measured at the isosbestic wavelength, the rate of fluorescence decrease is insensitive to changes in $[Ca^{2+}]_i$ and is proportional to the rate of Mn^{2+} influx [18].

Incubation with oxidized ATP (oATP)

Leydig cells isolated as described above were preincubated for 2 h in the presence of 0.3 mM oATP as previously described [19]. Cells were then washed and resuspended in standard saline, and treated with various nucleotides (100 μM final concentration for 3 h) for evaluation of testosterone secretion. For $[Ca^{2+}]_i$ determination after incubation with oATP, cells were loaded with fura-2/AM and $[Ca^{2+}]_i$ determined as described above.

Measurement of plasma membrane permeability

Increases in plasma membrane permeability were monitored by measuring ethidium bromide uptake. Leydig cell suspensions were placed in a thermostatted and magnetically stirred fluorimeter cuvette containing standard saline plus 20 μM ethidium bromide. ATP was added at a final concentration of 100 μM, and fluorescence changes were monitored at the wavelength pair 360/580 nm as described by Murgia et al. [19].

Hormone measurements

Testosterone was determined by radioimmunoassay, using [^{3}H]testosterone (Radim, Rome, Italy), as previously described [20]. The sensitivity of the assay was estimated as 0.28 nmol/l, and the intra- and inter-assay coefficients of variation were 7.8 % and 7.0 % respectively.

Statistical analysis

Results of five independent experiments were obtained and are expressed as means ± S.D. Statistical analysis was carried out using analysis of variance (ANOVA), Duncan's Multiple Range Test and Student's *t* test for unpaired data. A *P* value of less than 0.05 was chosen as the limit for statistical significance.

RESULTS

Effects of ATP on testosterone secretion

We first investigated the effects of extracellular ATP on testosterone secretion. ATP stimulated testosterone production in a dose- and time-dependent manner, with maximal effects at 100 μM and after 3 h of incubation (Figures 1 and 2). At higher doses and longer incubation times testosterone production did not increase (Figures 1A and 2). ATP-stimulated testosterone secretion was dependent on extracellular Ca^{2+}, as it was completely inhibited by removal of this cation from the incubation medium (Figure 1B).

Effects of ATP on $[Ca^{2+}]_i$ in rat Leydig cells

Extracellular ATP is known to increase $[Ca^{2+}]_i$ in a number of different cell types. Figure 3 shows that, in the presence of extracellular Ca^{2+} (1.7 mM), ATP caused a rapid and dose-dependent elevation of $[Ca^{2+}]_i$ in Leydig cells. The lowest effective concentration was greater than 1.0 μM and the maximal effect was observed at 100 μM (Figure 3B). The rise in $[Ca^{2+}]_i$ was biphasic, with a transient peak followed by a sustained plateau; the latter was dependent on Ca^{2+} influx from the extracellular medium, since it was obliterated by chelation of the extracellular cation by EGTA (Figure 3A). The first rapid phase of the increase in $[Ca^{2+}]_i$ was due to release of intracellular Ca^{2+} stores,

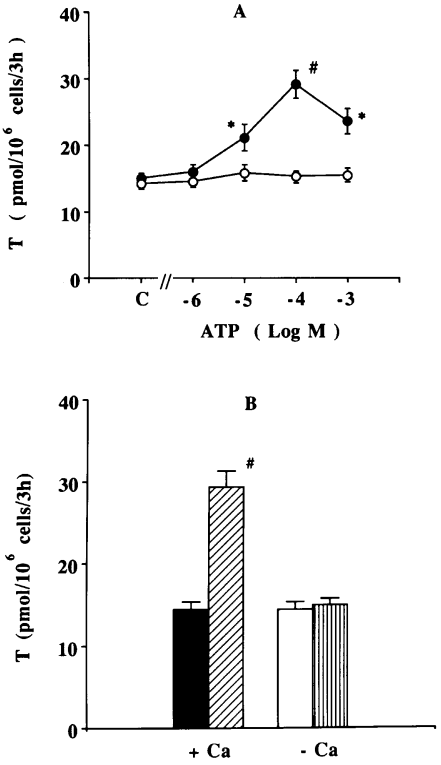

Figure 1 Extracellular ATP stimulates testosterone production in rat Leydig cells

(**A**) Adult rat Leydig cells, isolated as described in the Experimental section, were treated with ATP (●) or vehicle alone (○) in the presence of extracellular Ca^{2+}. (**B**) Dependence of extracellular-ATP-stimulated testosterone secretion on the presence of Ca^{2+} in the extracellular medium (left-hand bar of each pair, vehicle; right-hand bars, +100 μM ATP). Results are means ± S.D. of five separate experiments; $*P < 0.01$; $\#P < 0.001$ compared with control (vehicle). T, testosterone; C, control.

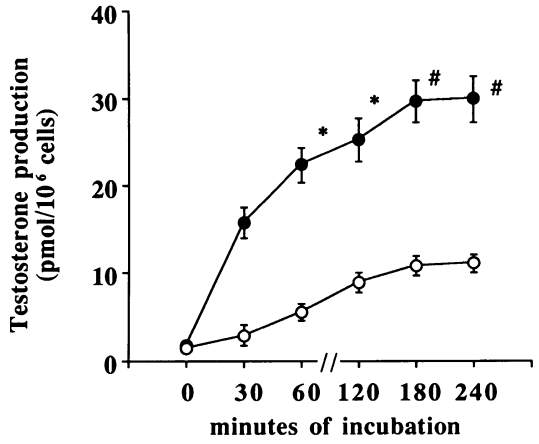

Figure 2 Time course of the effects of extracellular ATP on testosterone production in rat Leydig cells

Adult rat Leydig cells, isolated as described in Experimental section, were treated with 100 μM ATP (●) or vehicle alone (○). Results are means ± S.D. of three separate experiments; $*P < 0.01$; $\#P < 0.001$ compared with control (vehicle).

since it also occurred in the absence of extracellular Ca^{2+} (Figure 4A). Interestingly, when Ca^{2+} was added back to the Ca^{2+}-free medium, the sustained plateau observed in the presence of

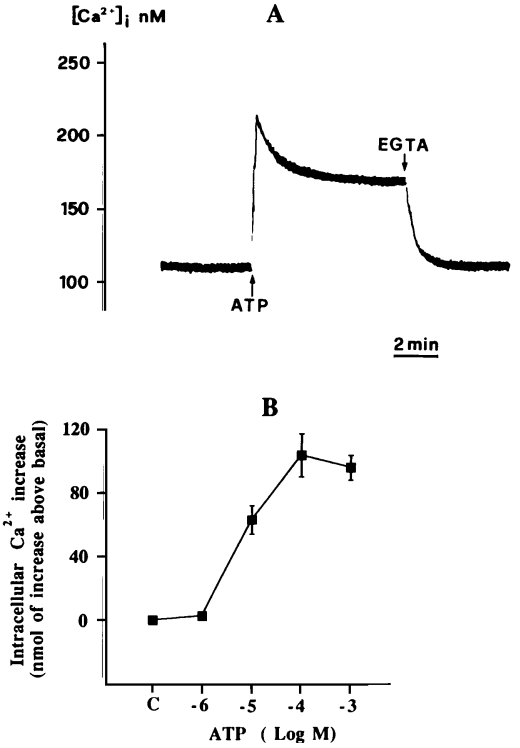

Figure 3 Extracellular ATP increases $[Ca^{2+}]_i$ in rat Leydig cell suspensions

Isolated adult rat Leydig cells were loaded with fura-2/AM as described in the Experimental section. (**A**) Representative trace for the ATP-induced rise in $[Ca^{2+}]_i$ in a typical experiment of five. Where indicated, 100 μM ATP was added. (**B**) Dose–response relationship: initial peak $[Ca^{2+}]_i$ increases above the basal level are plotted against ATP concentration. Results are means ± S.D. of triplicate determinations from five separate experiments.

external Ca^{2+} was restored. Furthermore, inhibition of the ATP-dependent rise in $[Ca^{2+}]_i$ in Ca^{2+}-free medium by thapsigargin, an inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase [21], suggests that ATP mobilizes Ca^{2+} from the endoplasmic reticulum (Figure 4B).

Effects of ATP on Mn^{2+} uptake

The ability of Mn^{2+} to quench fura-2 fluorescence at a Ca^{2+}-insensitive wavelength (360 nm) can be taken as further proof of ATP-dependent Ca^{2+} influx. At this excitation wavelength ATP resulted in a rapid decrease in fluorescence, consistent with rapid Mn^{2+} influx (Figure 5).

Effects of ATP on plasma membrane permeability

Changes in plasma membrane permeability were monitored by measuring increases in ethidium bromide fluorescence. Leydig cell suspensions incubated in the presence of 20 μM ethidium bromide did not show any increase in plasma membrane permeability after challenge by 100 μM ATP (results not shown).

Effects of other nucleotides

We then examined the effects of other extracellular nucleotides on testosterone secretion and $[Ca^{2+}]_i$. Of the different nucleotides tested (final concentration 100 μM), only ATP, adenosine 5′-[γ-thio]triphosphate (ATP[S]), benzoylbenzoic-ATP (BzATP),

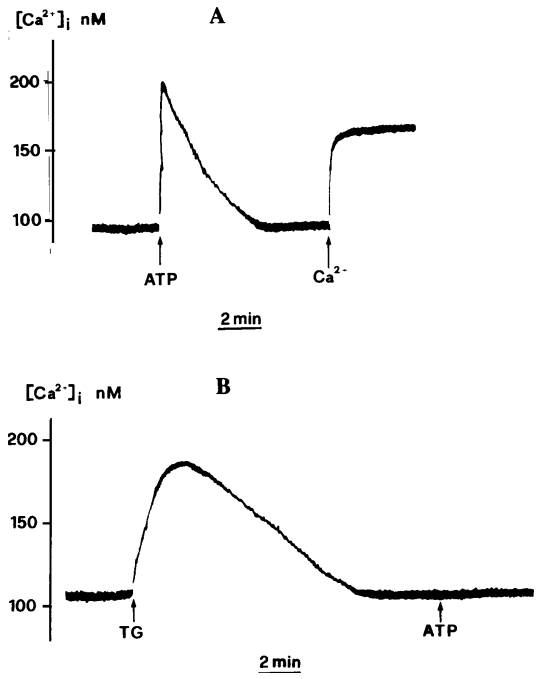

Figure 4 Extracellular ATP induces Ca^{2+} mobilization from internal stores in rat Leydig cells

Isolated adult rat Leydig cells were loaded with fura-2/AM as described in the Experimental section, and then resuspended in Ca^{2+}-free medium (no added Ca^{2+}; 0.1 mM EGTA). (**A**) Leydig cells were treated with 100 μM ATP, and then 1.8 mM Ca^{2+} was added. (**B**) Leydig cells were pretreated with 100 nM thapsigargin before addition of ATP (100 μM) that started as $[Ca^{2+}]_i$ returned to resting values. Results are representative of five separate similar experiments.

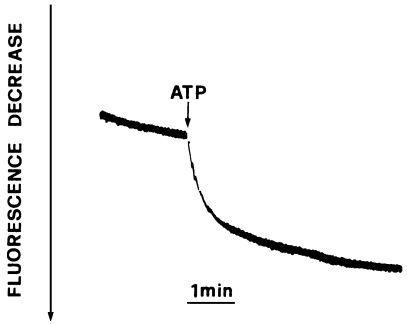

Figure 5 Extracellular ATP stimulates Mn^{2+} uptake in rat Leydig cells

Isolated adult rat Leydig cells were loaded with fura-2/AM as described in the Experimental section, and resuspended in medium containing $MnCl_2$ (200 μM). Fluorescence was monitored at the Ca^{2+}-insensitive excitation wavelength (360 nm). Where indicated, ATP (100 μM) was added. The trace is representative of three similar experiments.

2-methylthio-ATP (2-MeSATP) and UTP were effective. ATP, ATP[S] and UTP were about equipotent with respect to both testosterone production and $[Ca^{2+}]_i$ increase. BzATP and 2-MeSATP were much more potent than ATP and, rather interestingly, the mechanisms of activation appeared to be different: 2-MeSATP (like ATP) caused both Ca^{2+} influx and release from stores; on the contrary, BzATP only caused Ca^{2+} influx from the extracellular medium, with no release from intracellular stores (Table 1). Both the $[Ca^{2+}]_i$ increase and

Table 1 Effects of different nucleotides on testosterone production and $[Ca^{2+}]_i$ in rat Leydig cells

Isolated adult rat Leydig cells were treated as described in the Experimental section with the different nucleotides, each at a final concentration of 100 μM, in the presence and absence of extracellular Ca^{2+} (1.7 mM). Values are means $\pm$ S.D. of three separate experiments; $^*P < 0.01$, $^{**}P < 0.001$ compared with control. Abbreviations: AMP-CPP, adenosine 5′-(α,β-methylene)triphosphate; AMP-PCP, adenosine 5′-[β,γ-methylene)triphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate.

	Testosterone production (pmol/3 h per 10^6 cells)		Peak $[Ca^{2+}]_i$ rise (nmol above basal)	
Nucleotide	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
Control	13.8 $\pm$ 1.4	14.1 $\pm$ 1.5	0	0
ATP	29.3 $\pm$ 2.0*	15.8 $\pm$ 1.2	103.5 $\pm$ 13.4**	101.1 $\pm$ 12.7**
ATP[S]	32.2 $\pm$ 2.1*	16.0 $\pm$ 1.5	107.7 $\pm$ 13.9**	103.7 $\pm$ 10.6**
UTP	28.4 $\pm$ 1.8*	15.4 $\pm$ 1.9	104.5 $\pm$ 10.9**	100.9 $\pm$ 9.7**
BzATP	45.6 $\pm$ 4.1**	17.4 $\pm$ 2.3	207.3 $\pm$ 19.1**	0
MeSATP	45.8 $\pm$ 3.9**	17.5 $\pm$ 2.1	193.8 $\pm$ 17.3**	35.8 $\pm$ 7.9*
AMP-CPP	16.1 $\pm$ 1.9	15.5 $\pm$ 1.3	6.0 $\pm$ 0.7	0
AMP-PCP	14.7 $\pm$ 1.6	15.7 $\pm$ 1.1	2.6 $\pm$ 0.3	0
CTP	13.9 $\pm$ 1.2	14.2 $\pm$ 1.3	0	0
GTP	14.0 $\pm$ 1.3	14.9 $\pm$ 1.7	0	0
GTP[S]	13.9 $\pm$ 1.5	14.5 $\pm$ 1.5	0	0

Table 2 oATP inhibits the rise in $[Ca^{2+}]_i$ and testosterone production induced by different nucleotides

Leydig cells, isolated as described in the Experimental section, were preincubated for 2 h in the presence of 0.3 mM oATP. Cells were then washed and resuspended in standard saline, and stimulated with different nucleotides for 3 h (100 μM final concentration) for the determination of testosterone secretion and $[Ca^{2+}]_i$, as described in the Experimental section. Values are means $\pm$ S. D. of three separate experiments.

Nucleotides	Testosterone production (pmol/3 h per 10^6 cells)	Peak $[Ca^{2+}]_i$ rise (nmol above basal)
oATP	14.8 $\pm$ 1.9	0
oATP + ATP	15.9 $\pm$ 1.4	8.1 $\pm$ 0.7
oATP + BzATP	16.7 $\pm$ 1.1	0
oATP + MeSATP	16.5 $\pm$ 1.6	11.9 $\pm$ 4.0

testosterone production stimulated by ATP were blocked by preincubation in the presence of the P2Z receptor blocker oATP (Table 2).

DISCUSSION

Increasing evidence supports a role for extracellular ATP in the regulation of gonadal function. In humans, this nucleotide stimulates the sperm acrosome reaction and fertilization by activating a P2-purinergic receptor coupled to an ion channel permeable to Na^+ [17]. In human and pig granulosa cells this nucleotide stimulates progesterone and oestradiol secretion, acting at P2-purinergic receptors coupled to intracellular Ca^{2+} mobilization and Ca^{2+} influx [22]. Furthermore, extracellular ATP modulates rat Sertoli cell activity through activation of different P2-purinergic receptor subtypes [23,24].

The results of the present study provide the first evidence for the presence of P2-purinergic receptors in rat Leydig cells. When activated by ATP, these receptors trigger an increase in $[Ca^{2+}]_i$ that displays two components: an initial rapid rise, dependent on the depletion of intracellular Ca^{2+} stores, followed by a sustained and prolonged phase of $[Ca^{2+}]_i$ elevation that is dependent on

influx of Ca^{2+} from the extracellular medium. The inhibition of the initial $[Ca^{2+}]_i$ increase by depletion of intracellular Ca^{2+} stores with thapsigargin supports the hypothesis that ATP induces release of internal Ca^{2+} stores. Furthermore, the present findings show that extracellular ATP stimulates rat Leydig cell steroidogenesis in a time- and dose-dependent manner. Analysis of the Ca^{2+}-dependence of testosterone secretion shows that stimulation of the latter by ATP is mainly, if not exclusively, mediated by Ca^{2+} influx from the extracellular medium, since when external Ca^{2+} was absent, testosterone production was completely inhibited. The role of the release of internal Ca^{2+} stores induced by ATP is unclear, and it could be related to non-secretory events.

Purinergic receptors are classified into P1 and P2 types, responsive to adenosine and ATP respectively [15]. The possibility that the effects of ATP in Leydig cells are due to activation of P1 receptors by ATP degradation products can be ruled out, since AMP and adenosine have no effect on $[Ca^{2+}]_i$ and testosterone secretion in Leydig cells [25]. Furthermore, the fact that the non-hydrolysable analogue ATP[S] is also a stimulant confirms that ATP hydrolysis is not necessary and that P1-purinergic receptors are not involved.

According to pharmacological criteria, P2-purinergic receptors are grouped into different families (P2T, P2Y, P2U, P2X and P2Z), each comprising several members [15]. P2T receptors are only present in platelets and are coupled to activation of a cation-selective channel. P2Y receptors (of which P2U is a member) are seven-membrane-spanning receptors that are coupled to G-proteins and Ca^{2+} mobilization from internal stores [26]. P2X receptors act as ligand-gated ion channels, and P2Z receptors are associated with the formation of non-specific membrane pores permeable to solutes of less than 900 Da [15]. Since there are no previous data available on Leydig cells, the identification of the P2-purinergic receptor subtype expressed by these cells is still very preliminary. P2T-purinergic receptors can be ruled out, since they are present only on platelets and ATP is an antagonist rather than an agonist. Typical P2Z receptors can also be excluded, because we observed no sign of increased plasma membrane permeability to low-molecular-mass extracellular molecules, a hallmark of P2Z receptor activation [15]. P2Y receptors are linked to the release of Ca^{2+} from intracellular stores. Stimulation by ATP in Leydig cells was consistently coupled with the thapsigargin-sensitive release of Ca^{2+} from intracellular stores, an effect also shared by UTP. These data support the presence of the P2Y-purinergic receptor subtype in rat Leydig cells, although the direct involvement of this receptor subtype in ATP-stimulated steroidogenesis remains to be demonstrated.

Structural and functional evidence suggest that P2Y-and P2U-purinoreceptor subtypes, whether activated by ATP or UTP, belong to the same family of receptors [26]. At present, however, we do not know whether Leydig cells express two separate receptors for ATP and UTP or a single receptor with two separate binding sites. While the activation of the putative P2Y/U receptor in Leydig cells may be responsible for the rapid $[Ca^{2+}]_i$ rise induced by ATP that also occurs in Ca^{2+}-free medium, the nature of the sustained Ca^{2+} influx is less clear. Ca^{2+} influx from the extracellular space, activated by ATP, could be due to activation of a P2X-purinergic receptor subtype, i.e. a ligand-gated channel that is permeable to cations (Ca^{2+}, Na^+, K^+). This putative P2X receptor expressed on Leydig cell plasma membranes appears to have a distinct pharmacological profile, since a well known potent agonist of this receptor subtype, adenosine 5′-[α,β-methylene]triphosphate, was only weakly effective. However, a P2X receptor with a similar pharmacological profile has been cloned from PC12 cells [27]. Thus it is possible that the sustained Ca^{2+} influx is due, at least in part, to activation of this receptor subtype. However, rather intriguingly, the ATP-gated channel in Leydig cells was also activated by BzATP and blocked by oATP, two agents that are well known for their specificity for P2Z receptors. Thus it appears that Leydig cells express an ATP-gated plasma membrane channel that shares the pharmacological properties of P2X- and P2Z-purinergic receptors.

It is well known that extracellular ATP mediates a variety of distinct effects in many different cell types, including testicular cells [17,24]. The physiological role of ATP and P2-purinergic receptor activation in the modulation of Leydig cell function *in vivo* is unknown. In this respect, it will be of interest to identify a source of ATP that could activate P2-purinergic receptors on Leydig cells, since this nucleotide is normally present at very low concentrations in the extracellular space. ATP can be released from some cell types via secretory exocytosis or intrinsic plasma membrane channels without cell damage, but can also be released as a consequence of cellular breakage [15]. Furthermore, ATP is released *in vivo* during neurotransmitter release from cholinergic, adrenergic and purinergic nerve endings [28]. Physiological studies have indicated that testosterone secretion from Leydig cells is modulated by the autonomic nervous system both in humans and in other animals [7]. Autonomic nerve fibres have been described close to and in direct contact with Leydig cells, and have been demonstrated to be cholinergic and adrenergic [29,30]. It is possible that ATP released from these nerve endings modulates Leydig cell function.

In conclusion, the results of the present study show that rat Leydig cells possess P2-purinergic receptors and that activation of these receptors by extracellular ATP stimulates testosterone secretion via a mechanism dependent on the influx of Ca^{2+} from the extracellular medium. The availability of specific antagonists and the cloning of the different P2-purinergic receptors will help to identify the receptor subtypes expressed on Leydig cells.

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REFERENCES

1 Cooke, B. A., Choi, M. C. K., Dirami, G., Lopez-Ruin, M. P. and West, A. P. (1992) J. Steroid. Biochem. Mol. Biol. **43**, 445–449
2 Foresta, C. and Mioni, R. (1988) Arch. Androl. **4**, 101–107
3 Foresta, C., Mioni, R., Bordon, P., Gottardello, F., Nogara, A. and Rossato, M. (1995) Eur. J. Endocrinol. **132**, 103–108
4 Mioni, R., Gottardello, F., Bordon, P., Montini, G. and Foresta, C. (1992) Acta Endocrinol. **127**, 459–465
5 Ulisse, S., Fabbri, A. and Dufau, M. L. (1988) J. Biol. Chem. **264**, 2156–2163
6 Nieschlag, E., Baselt, P. and Cuppers, H. J. (1977) Acta Endocrinol. (Suppl. 212) **120**, abstract 187
7 Tinajero, J. C., Fabbri, A. and Dufau, M. L. (1993) Endocrinology (Baltimore) **133**, 257–264
8 Bertrand, G., Chapal, J. and Loubatieres-Mariani, M. M. (1986) Am. J. Physiol. **251**, E416–E421
9 Blachier, F. and Malaisse, W. J. (1988) Biochim. Biophys. Acta **970**, 222–229
10 Loubatieres-Mariani, M. M. and Chapal, J. (1988) Diabete Metab. **14**, 119–126
11 Davidson, J. S., Wakefield, I. K., Sohnius, U., Van Der Merwe, P. A. and Millar, R. P. (1990) Endocrinology (Baltimore) **126**, 80–87
12 Raspe, E., Andry, G. and Dumont, J. E. (1989) J. Cell. Physiol. **140**, 608–614
13 Majid, M. A., Okajima, F. and Kondo, Y. (1992) Biochim. Biophys. Acta **1136**, 283–289
14 Kawamura, M., Matsui, T., Niitsu, A., Kondo, T., Ohno, Y. and Nakamici, N. (1991) Jpn. J. Pharmacol. **56**, 543–545
15 Dubyak, G. R. and El-Moatassim, C. (1993) Am. J. Physiol. **265**, C577–C606
16 Mendelson, C., Dufau, M. L. and Catt, K. (1975) J. Biol. Chem. **250**, 8818–8823
17 Foresta, C., Rossato, M. and Di Virgilio, F. (1992) J. Biol. Chem. **267**, 19443–19447
18 Alvarez, J., Montero, M. and Garcia-Sancho, J. (1991) Biochem. J. **274**, 193–197

19 Murgia, M., Hanau, S., Pizzo, P., Rippa, M. and Di Virgilio, F. (1993) J. Biol. Chem. **268**, 8199–8203
20 Foresta, C., Ruzza, G., Rizzotti, A., Lembo, A., Valente, M. L. and Mastrogiacono, I. (1984) J. Androl. **5**, 135–137
21 Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2466–2470
22 Kamada, S., Blackmore, P. F., Oehninger, S., Gordon, K. and Hodgen, G. D. (1994) J. Clin. Endocrinol. Metab. **78**, 650–656
23 Filippini, A., Riccioli, A., De Cesaris, P., Paniccia, R., Teti, A., Stefanini, M., Conti, M. and Ziparo, E. (1994) Endocrinology (Baltimore) **134**, 1537–1545
24 Foresta, C., Rossato, M., Bordon, P. and Di Virgilio, F. (1995) Biochem. J. **311**, 269–274
25 Sullivan, M. H. and Cooke, B. A. (1986) Biochem. J. **236**, 45–51
26 Lustig, K. D., Shiau, A. K., Brake, A. J. and Julius, D. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 5113–5117
27 Brake, A. J., Wagenbach, M. J. and Julius, D. (1994) Nature (London) **371**, 519–522
28 Burnstock, G. (1971) Nature (London) **229**, 282–283
29 Prince, F. P. (1992) Cell Tissue Res. **269**, 383–390
30 Rauchenwald, M., Steers, W. D. and Desjardens, C. (1995) Biol. Reprod. **52**, 1136–1143

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