Extracellular ATP activates different signalling pathways in rat Sertoli cells

Carlo FORESTA,*†, Marco ROSSATO,* Paola BORDON* and Francesco DI VIRGILIO†

*Institute of Internal Medicine, University of Padova, Via Ospedale 105, 35128 Padova, Italy, and †Institute of General Pathology, University of Ferrara, Via Borsari 46, 44100 Ferrara, Italy

1. The present study describes effects of extracellular ATP (ATP_e) on plasma membrane potential and cytoplasmic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) in rat Sertoli cells. Sertoli cells in suspension were stimulated with ATP_e and other nucleotides and ionic changes were monitored utilizing the fluorescent dyes bis-oxonol and fura-2/AM. ATP_e induced a prompt plasma membrane depolarization which was dependent on Na^+ influx from the extracellular medium, since it was abolished by omission of extracellular Na^+ . Depolarization was independent of $[\text{Ca}^{2+}]_i$ rise as it also occurred in the absence of extracellular Ca^{2+} and after intracellular Ca^{2+} stores were discharged with thapsigargin. ATP_e also stimulated a rapid and biphasic increase in $[\text{Ca}^{2+}]_i$: a prompt spike was followed by a prolonged sustained plateau. The initial spike was dependent on Ca^{2+} release from intracellular stores since it was also present when cells were incubated in EGTA-supplemented Ca^{2+} -free medium and was abolished by pretreatment with ionomycin and thapsigargin, agents that discharge intracellular Ca^{2+} stores. The sustained phase was dependent on Ca^{2+} influx from the extracellular medium as it was abolished

when cells were incubated in EGTA-supplemented Ca^{2+} -free medium. Ca^{2+} influx was due to activation of voltage-operated calcium channels (VOCCs) since it was abolished by the VOCC inhibitors verapamil and nifedipine or incubation in sucrose medium, an experimental condition which precludes plasma membrane depolarization by ATP_e . 2. ATP_e -induced rises in intracellular Ca^{2+} concentration and plasma membrane depolarization were reduced by pretreatment with pertussis toxin, suggesting that ATP_e -activated transduction mechanisms are in part under the control of pertussis toxin-sensitive G-proteins. These data show that Sertoli cells possess P2-purinergic receptor subtypes coupled to influx of Na^+ and release of Ca^{2+} from intracellular stores and provide evidence for an activation of different pathways by extracellular ATP_e . Activation of these receptors induces Na^+ influx that causes a rapid plasma membrane depolarization. Furthermore, ATP_e also triggers Ca^{2+} release from intracellular stores and Ca^{2+} influx from extracellular space via dihydropyridine-sensitive VOCCs.

INTRODUCTION

In recent years there has been a rekindled interest in the role of extracellular ATP (ATP_e) in the modulation of cellular functions (reviewed in [1]). It has been shown that ATP_e elicits biological responses in many different tissues: it modulates contractility of vascular smooth muscle cells [2] and cardiac myocytes [3], acts as a neurotransmitter in the nervous system [4] and plays an important role in the immune and endocrine-exocrine systems [5–7]. Plasma membrane receptors for ATP_e are called P2-purinergic receptors and according to pharmacological and structural criteria can be grouped in three main families (P2Y, P2X and P2Z), each comprising several members. The P2Y family, of which P2U is a numbered subtype [8], identifies seven membrane-spanning receptors that act as G-protein-coupled calcium-mobilizing receptors [9]. P2X receptors are a family of ligand-gated ion channels. The P2Z receptor is associated with the opening of non-specific membrane pores permeable to solutes of less than 900 Da.

Little is known about the role of ATP_e in the male reproductive system and in the regulation of germ and Sertoli cell functions, apart from recent studies reporting that ATP_e is a potent inducer of the acrosome reaction in human spermatozoa activating a putative novel P2X receptor subtype coupled to Na^+ influx [10].

It has long been known that rat Sertoli cells in culture possess adenosine receptors coupled to G_i and to inhibition of adenylate cyclase [11,12]. Only recently, however, has it also been shown that rat Sertoli cells possess P2-purinergic receptors, and that

ATP_e (and UTP), probably acting at the P2-purinergic receptor subtype P2U, is able to trigger phosphoinositide (PI) turnover and intracellular calcium ($[\text{Ca}^{2+}]_i$) changes [13]. In Sertoli cells, ATP_e triggers both Ca^{2+} release from intracellular stores and Ca^{2+} influx across the plasma membrane, suggesting that different transduction systems may be activated [13]. In the present study we have investigated the mechanisms of ATP_e -induced intracellular ion changes. Our results on the one hand confirm that ATP_e mobilizes Ca^{2+} from intracellular stores in immature Sertoli cells, and on the other show that this nucleotide also induces a selective Na^+ influx from the extracellular medium, probably activating a novel P2X-receptor subtype. The ensuing plasma membrane depolarization causes opening of voltage-gated Ca^{2+} channels (VOCCs) that contribute to the sustained $[\text{Ca}^{2+}]_i$ plateau following the initial fast $[\text{Ca}^{2+}]_i$ transient.

Our results suggest that ATP_e stimulates in Sertoli cells two separate transduction systems that synergize in causing an elevated $[\text{Ca}^{2+}]_i$.

MATERIALS AND METHODS

Isolation of Sertoli cells

Testes were isolated from 18-day-old Sprague–Dawley rats and placed in Dulbecco's modified Eagle's medium (DMEM). After decapsulation, testes were digested utilizing a modification of the procedure developed by Gorczynska and Handelsman [14]. Briefly, testes were placed in DMEM containing trypsin (2.5 g/l),

Abbreviations used: ATP_e , extracellular ATP; ATP[S] , adenosine-5'-O-[3-thio]triphosphate; GTP[S] , guanosine-5'-O-[3-thio]triphosphate; AMP-CPP, α,β -methylene-ATP; AMP-PCP, adenosine-5'-[β,γ -methylene]-triphosphate; 2MeSATP, 2-methylthioadenosine-5'-triphosphate; AMP-PNP, adenylyl-5'-yl imidodiphosphate; PI, phosphoinositides; $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentrations; VOCCs, voltage-operated calcium channels; FSH, follicle-stimulating hormone; DMEM, Dulbecco's modified Eagle's medium.

† To whom correspondence should be addressed.

collagenase (1.0 g/l) and DNase (20 mg/l) for 30 min at 32 °C in a shaking water bath (60 cycles/min). Sertoli cells were then washed and centrifuged three times at 25 *g* for 2 min at 22 °C in DMEM containing DNase (10 mg/l). Finally cells were diluted appropriately in DMEM and counted. These cell preparations contain about 95 % Sertoli cells identified by light microscopy.

The accuracy of Sertoli cell isolation was determined by the demonstration that follicle-stimulating hormone (FSH) was able to induce an increase of $[Ca^{2+}]_i$ in suspended Sertoli cells, as previously demonstrated by Gorcynska and Handelsman [14] (results not shown).

Sertoli cells, isolated as above, were then either plated on glass rectangular coverslips (24 mm × 10 mm) in Petri dishes or kept in suspension in polypropylene tubes at 37 °C. The Sertoli cell suspensions were utilized within 5 h. Monolayers were grown in DMEM (supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone) at 32 °C in a controlled atmosphere of 95 % O₂ and 5 % CO₂. Medium was replaced every day. On day 3 of culture, contaminating germ cells were lysed by treatment with a hypotonic solution as described by Galdieri et al. [15]. After 24 h of recovery, cell monolayers were utilized for experiments.

Measurement of $[Ca^{2+}]_i$ and plasma membrane potential in Sertoli cell monolayers

Sertoli cell monolayers were incubated with the fluorescent probe fura-2/AM (2 µM) for 45 min at 37 °C. After loading, medium was discarded and replaced with fresh DMEM and the monolayers were kept at room temperature until used. For $[Ca^{2+}]_i$ measurement, the coverslips were placed into the fluorimeter cuvette filled with 2 ml of a standard saline [Biggers, Whittam and Whittingham (BWW); containing in mM: 95, NaCl; 4.8, KCl; 1.2, MgSO₄; 1.2, KH₂PO₄; 5.6, fructose; 25, NaHCO₃; 1.7, CaCl₂; 0.25, sodium pyruvate] and 3.7 ml/l sodium lactate syrup (60 %), 10⁴ i.u./ml penicillin, 10 mg/ml streptomycin and 20 Hepes, pH 7.4, at 37 °C at an angle of 30 ° to the excitation light beam. Coverslips were kept in this position by a Teflon coverslip holder as previously described in detail [16]. This device prevented contact of the coverslip with the stirring bar placed on the bottom of the cuvette, thus allowing medium stirring. Two ports present in the Teflon holder allowed additions of different agents to the medium, thus avoiding repeated openings of the cuvette.

For measurement of plasma membrane potential in Sertoli cell monolayers, coverslips were placed in the fluorimeter cuvette containing 200 nM bis-oxonol.

Measurement of $[Ca^{2+}]_i$ and plasma membrane potential in Sertoli cell suspensions

Sertoli cell $[Ca^{2+}]_i$ was measured with the fluorescent probe fura-2/AM as previously described [14]. Briefly, Sertoli cell suspensions were incubated in BWW supplemented with 0.3 % BSA in the presence of 10 µM fura-2/AM for 45 min at 37 °C. After loading, cells were washed free of extracellular dye by being centrifuged three times (25 *g* for 2 min at room temperature) in standard saline supplemented with 0.3 % BSA and maintained at room temperature until used. All experiments were completed within 3 h of loading with fura-2. $[Ca^{2+}]_i$ was determined as previously described [10].

Plasma membrane potential changes were monitored with the lipophilic membrane potential-sensitive dye bis-oxonol at the wavelength pair 540/580 nm as previously described [17].

In some experiments NaCl was replaced by an iso-osmotic concentration of choline chloride, methylglucamine or sucrose, as previously described [17].

Sertoli cell plasma membrane permeability was monitored by measuring ethidium bromide uptake, as previously described [18]. Sertoli cells in suspension were transferred to a thermostatically controlled and magnetically stirred fluorimeter cuvette containing 20 µM ethidium bromide. Fluorescence changes were monitored at the wavelength pair 360/580 nm.

Since results obtained utilizing monolayers and suspensions of Sertoli cells were similar, the majority of the experiments were performed with suspended Sertoli cells.

Statistical analysis

Data were analysed utilizing the Stat View (Abacus Concepts, Berkely, CA, U.S.A.) statistical package. Student's *t* test was used for statistical evaluation.

Chemicals

ATP and all other nucleotides were purchased from Boehringer Mannheim (Mannheim, Germany). Pertussis toxin, gramicidin D, thapsigargin, trypsin, collagenase and DNase were obtained from Sigma (St. Louis, MO, U.S.A.). DMEM was purchased from Gibco (Grand Island, NY, U.S.A.). Fura-2/AM and bis-oxonol were obtained from Molecular Probes (Eugene, OR, U.S.A.). Ionomycin was purchased from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals were of analytical grade.

RESULTS

Effect of ATP_e on Sertoli cell plasma membrane potential

Activation of plasma membrane receptors for ATP_e is known to induce modifications in intracellular ion homeostasis and membrane permeability to solutes of low molecular mass, and to cause changes in plasma membrane potential [19]. As shown in Figure 1, ATP_e triggers a rapid (Figure 1a) and dose-related (Figure 1b) plasma membrane depolarization, with a minimal efficacious dose of 0.5 µM and an EC₅₀ of ~ 10 µM. This effect was not maximal since addition of gramicidin D caused a further collapse in plasma membrane potential. ATP_e-induced depolarization was not dependent on the presence of Ca²⁺ in the extracellular medium as it was not obliterated by removal of extracellular Ca²⁺ (Ca²⁺-free medium plus 0.1 mM EGTA) (Fi-

Figure 1 Effects of ATP_e on plasma membrane potential

(a) Sertoli cells in suspension [(5.0–7.5) × 10⁵ cells/ml] were incubated in the presence of 1.7 mM Ca²⁺ and 1.2 mM Mg²⁺. Where indicated, ATP (100 µM) and gramicidin D (Gram; 0.1 µg/ml) were added. (b) Dose-dependent effects of ATP_e on plasma membrane potential of Sertoli cells in the presence of 200 nM bis-oxonol. Depolarization is expressed as arbitrary units of fluorescence increase. Data are means ± S.D. of triplicate determinations from five separate experiments.

Figure 2 ATP_e-induced plasma membrane depolarization does not depend on intracellular Ca²⁺ stores depletion

Sertoli cells $[(5.0-7.5) \times 10^5 \text{ cells/ml}]$ were suspended in Ca²⁺-free medium (no added Ca²⁺ and 0.1 mM EGTA) and stimulated with ATP_e in the absence (a) and in the presence (b) of thapsigargin. Additions were: thapsigargin (Tg), 100 nM; Ca²⁺, 1.8 mM; ATP, 100 μM ; KCl, 30 mM. Results are representative of five similar experiments.

Figure 3 Effects of ATP_e on plasma membrane potential in the absence of extracellular Na⁺

Sertoli cells $[(5.0-7.5) \times 10^5 \text{ cells/ml}]$ were suspended in Na⁺-containing saline (trace a) or in saline in which Na⁺ was iso-osmotically replaced by choline (trace b), methylglucamine (trace c) or sucrose (trace d) as described in the Materials and Methods section. Where indicated, ATP (100 μM) and KCl (15 mM) were added. Results are representative of three to five similar experiments.

Figure 2a). Furthermore, ATP_e was still capable of inducing depolarization after full discharge of the intracellular Ca²⁺ stores by thapsigargin (Figure 2b). It is of interest that the [Ca²⁺]_i rise induced by thapsigargin (cf. Figure 5b, trace b) was unable to depolarize the plasma membrane (Figure 2b). Since ATP_e is able to induce plasma membrane depolarization even in the absence of extracellular Ca²⁺, we investigated whether Na⁺ was the main ion permeable through the ATP_e-gated channel. Figure 3 shows that when extracellular Na⁺ was iso-osmotically replaced with choline or methylglucamine (cations less permeant than Na⁺ through the ATP_e channel [20], traces b and c respectively), ATP_e was still able to trigger a plasma membrane depolarization, although smaller than that in Na⁺ medium (Figure 3, trace a). Furthermore, iso-osmotic substitution of extracellular Na⁺ with sucrose (a completely impermeant solute through the ATP_e-gated channel) [20] prevented ATP_e-induced plasma membrane depolarization (trace d). Accordingly, in these substituted media, gramicidin D caused little or no depolarization, since these cations are poorly or not at all permeable through the gramicidin D pore; in these conditions, however, KCl was still able to depolarize the cells. Altogether these findings suggest that Na⁺ is

Figure 4 Effects of ATP_e on [Ca²⁺]_i Sertoli cells in suspension

Sertoli cells $[(5.0-7.5) \times 10^5 \text{ cells/ml}]$ were loaded with fura-2/AM as described in the Materials and methods section. (a) Representative trace for ATP_e-induced [Ca²⁺]_i rise in a typical experiment from five. Where indicated, ATP (100 μM) was added. (b) Dose-response relationship between peak [Ca²⁺]_i rise and ATP_e concentrations. Peak [Ca²⁺]_i increases above basal level were plotted against ATP_e concentrations. Means $\pm$ S.D. of duplicate determinations for five separate experiments are shown.

Figure 5 ATP_e induces Ca²⁺ mobilization from internal stores in rat Sertoli cells

Sertoli cells $[(5.0-7.5) \times 10^5 \text{ cells/ml}]$, loaded with fura-2/AM as described in the Materials and methods section, were suspended in standard Ca²⁺-free saline (no Ca²⁺ added and 0.1 mM EGTA). (a) Sertoli cells were treated with 100 μM ATP_e and then 1.8 mM Ca²⁺ was added. (b) Sertoli cells were pretreated with 1.0 μM ionomycin (Iono, trace a) or 100 nM thapsigargin (Tg, trace b) before ATP_e (100 μM) addition that started as [Ca²⁺]_i returned to resting values. Results are representative of five such experiments.

the main permeant ion through the ATP_e-gated channel in rat Sertoli cells and that other low-molecular-mass monovalent cations beside Na⁺ (choline and methylglucamine) can be admitted, as demonstrated in other cellular systems [17,20].

Effects of ATP_e on [Ca²⁺]_i in Sertoli cell suspensions

In many cell types the effects elicited by ATP_e are associated with an increase in cytosolic Ca²⁺ [21]. Figure 4(a) shows that in rat

Figure 6 Effects of ATP_e on Ca^{2+} influx from extracellular space

Sertoli cells $[(5.0\text{--}7.5) \times 10^5 \text{ cells/ml}]$, loaded with fura-2/AM, were suspended (a) in Na^+ -free saline (in which Na^+ was iso-osmotically replaced with sucrose) and (b) in standard saline containing 1.7 mM Ca^{2+} in the absence (trace a) or in presence (trace b) of 20 μM verapamil (Ver) or 1.0 μM nifedipine (Nif) added 5 min before ATP_e challenge. These two VOCC blockers gave similar results during different experiments. Where indicated ATP_e (100 μM) was added. Traces are representative of three similar experiments.

Sertoli cells this nucleotide caused a biphasic $[Ca^{2+}]_i$ rise: a prompt spike followed by a prolonged sustained plateau which lasted several minutes. The rise in $[Ca^{2+}]_i$ induced by ATP_e was dose-dependent, with a threshold concentration between 1.0 and 5.0 μM and an EC_{50} of 10 μM (Figure 4b). The kinetics of $[Ca^{2+}]_i$ rise were typical for activation of receptors that induces both Ca^{2+} influx and Ca^{2+} release from intracellular stores. In fact, when Ca^{2+} was omitted from the extracellular medium (0.1 mM EGTA and no Ca^{2+} added), ATP_e was still able to induce a rise of $[Ca^{2+}]_i$ that was transient, since the levels of $[Ca^{2+}]_i$ returned to basal levels in about 1 min (Figure 5a). Subsequent restoration of Ca^{2+} resulted in a restoration of the sustained elevation of $[Ca^{2+}]_i$. The sustained phase that follows the initial spike in $[Ca^{2+}]_i$ elicited by ATP_e is then clearly dependent on an influx from the extracellular medium. To further support the suggestion that ATP_e is able to mobilize Ca^{2+} from intracellular stores, we treated Sertoli cells suspended in Ca^{2+} -free medium with 1.0 μM ionomycin to deplete intracellular Ca^{2+} stores. Under these experimental conditions ATP_e was completely ineffective in raising $[Ca^{2+}]_i$ (Figure 5b, trace a). ATP_e was also unable to increase $[Ca^{2+}]_i$ when added after thapsigargin (100 nM) in Ca^{2+} -free medium; thapsigargin inhibits Ca^{2+} pumps of intracellular Ca^{2+} stores, thus causing a

Table 1 Effects of different nucleotides on plasma membrane potential and $[Ca^{2+}]_i$ in rat Sertoli cells

For plasma membrane potential and $[Ca^{2+}]_i$ measurement, Sertoli cells $[(5.0\text{--}7.5) \times 10^5 \text{ cells/ml}]$ were incubated with 200 nM bis-oxonol or loaded with fura-2/AM respectively, as described in the Materials and methods section. Experiments were performed in standard saline with 1.7 mM Ca^{2+} and 1.2 mM Mg^{2+} (pH 7.4 at 37 $^{\circ}\text{C}$). The effects on plasma membrane potential and $[Ca^{2+}]_i$ of 100 μM nucleotides are expressed as percentage of maximal depolarization induced by 100 μM ATP_e . Values given are averages of three experiments.

Nucleotide	Response	
	Depolarization (%)	$[Ca^{2+}]_i$ (%)
ATP	100	100
ATP[S]	95.2	88.5
UTP	82.3	91.7
GTP	6.5	0
GTP[S]	14.5	0
CTP	0	0
AMP-CPP	11.3	0
AMP-PCP	0	0
AMP-PNP	0	0
2MeSATP	0	0

direct discharge of intracellular Ca^{2+} stores without PI hydrolysis [22]. Thapsigargin added in Ca^{2+} -free medium induced a transient increase of $[Ca^{2+}]_i$. Following this response, the addition of ATP_e was completely ineffective (Figure 5b, trace b). Taken together, these findings suggest that ATP_e is able to mobilize Ca^{2+} from intracellular stores in rat Sertoli cells.

As reported above, presence of Na^+ in the extracellular medium was mandatory for ATP_e -induced membrane depolarization. Likewise, it is possible that Na^+ influx is necessary for ATP_e -triggered $[Ca^{2+}]_i$ elevations in Sertoli cells. ATP_e addition in sucrose medium induced a rise in $[Ca^{2+}]_i$ similar to that observed in the absence of extracellular Ca^{2+} , thus showing that only ATP_e -induced Ca^{2+} influx but not release from stores is dependent on Na^+ influx (Figure 6a). Since ATP_e is able to induce Na^+ influx and therefore plasma membrane depolarization (Figure 1a), we investigated whether influx of Ca^{2+} from extracellular medium occurs through VOCCs. Addition to the cell suspension of the Ca^{2+} -channel antagonists verapamil (20 μM) or nifedipine (1.0 μM) completely abolished the sustained plateau phase, demonstrating that it was depending on Ca^{2+} influx through VOCCs (Figure 6b).

Functional characterization of the ATP_e receptor is hindered by the absence of selective antagonists. We therefore examined the pharmacology of the purinergic receptor expressed on Sertoli cells, challenging these cells with different extracellular nucleotides. As shown in Table 1, besides ATP, only adenosine-5'-O-[3-thio]triphosphate (ATP[S]) and UTP stimulated Sertoli cell plasma membrane depolarization and $[Ca^{2+}]_i$ rise at micromolar concentrations and to an extent similar to that induced by ATP_e . GTP, guanosine-5'-O-[3-thio]triphosphate (GTP[S]), AMP-CPP, AMP-PCP, AMP-PNP and CTP had little or no effect. Finally, the effects of ATP, ATP[S] and UTP on plasma membrane depolarization and $[Ca^{2+}]_i$ were not additive (results not shown).

Effect of pertussis toxin on ATP_e triggered $[Ca^{2+}]_i$

Similar to many other receptors coupled to the mobilization of Ca^{2+} from intracellular stores, P2-purinergic agonist activation of phospholipase-C and the ensuing $InsP_3$ formation and Ca^{2+} mobilization are inhibited or attenuated by pertussis toxin [1,23].

Table 2 Pertussis toxin (PTX) reduces $[Ca^{2+}]_i$ rise and plasma membrane depolarization induced by ATP_e in rat Sertoli cells

Sertoli cells isolated as described in the Materials and methods section were incubated in the absence or presence of PTX (500 ng/ml) for 5 h in a controlled atmosphere (95% O₂/5% CO₂ at 37 °C). $[Ca^{2+}]_i$ and membrane potential measurements were performed in Ca²⁺-free medium as described in the Materials and methods section. Values are mean ± S.D. of three separate experiments with different ATP_e doses. Δ% represents percentage of reduction in ATP_e-induced $[Ca^{2+}]_i$ and depolarization peak in pertussis toxin-treated cells with respect to non-treated cells. Peak of ATP_e-induced plasma membrane depolarization is expressed as percentage of maximal effect induced by 0.1 μg/ml gramicidin D. n.d., not done.

ATP (mM)	Peak of $[Ca^{2+}]_i$ rise (nm above basal)			Peak depolarization (% of maximal induced by gramicidin)		
	Control	PTX	Δ%	Control	PTX	Δ%
0.01	56.7 ± 8.8	20.0 ± 4.8	−64.7	42.5 ± 2.1	21.5 ± 1.6	−49.4
0.1	97.4 ± 11.5	33.6 ± 15.1	−65.5	50.5 ± 1.7	28.0 ± 2.2	−44.6
1.0	78.4 ± 11.2	47.3 ± 7.2	−39.6	n.d.	n.d.	n.d.

In Table 2 it is shown that pretreatment of Sertoli cells with pertussis toxin induced a significant reduction (up to 65%) of ATP_e-induced $[Ca^{2+}]_i$ rise and plasma membrane depolarization.

DISCUSSION

There is substantial evidence for complex cell–cell interactions within the testis. These communications co-ordinate the activities of different testicular components to sustain an optimal spermatogenesis. Sertoli cells play a fundamental role in driving different phases of germ-cell maturation and it is well known that Sertoli cell activity is modulated by FSH. Interactions between myoid, interstitial and germ cells have been suggested even if little is known about the nature of the agents that mediate interactions between gonadal cells. In many different cellular systems it has been suggested that ATP_e could have a role as an extracellular mediator of cell-to-cell communication and the number of biological systems responsive to ATP_e is ever increasing [1].

In this study we confirm previous observations on the presence of P2-purinergic receptors in rat Sertoli cells and dissect the mechanism whereby this nucleotide perturbs intracellular ion homeostasis. In these cells ATP_e evoked a $[Ca^{2+}]_i$ rise that was also present in Ca²⁺-free medium, thus showing that this nucleotide is able to elicit release of Ca²⁺ from intracellular stores. These results fit very well with previous findings on ATP_e-stimulated PI breakdown. Inhibition of $[Ca^{2+}]_i$ increases after depletion of intracellular Ca²⁺ stores with ionomycin and thapsigargin further strengthens the hypothesis that internal Ca²⁺ stores are involved and that thapsigargin and ATP_e release Ca²⁺ from a common intracellular store [24,25].

Besides Ca²⁺ release from intracellular stores, ATP_e induced a prolonged Ca²⁺ influx from the extracellular medium that was inhibited in the presence of VOCC antagonists verapamil or nifedipine.

Our results show that ATP_e induces a fast plasma membrane depolarization due to Na⁺ influx from the extracellular space. This depolarization triggers VOCC activation and the subsequent Ca²⁺ influx that is responsible for the sustained plateau of $[Ca^{2+}]_i$ observed after ATP_e challenge. Accordingly, Ca²⁺ influx from extracellular medium was completely absent in Na⁺-free medium, an experimental condition in which ATP_e was unable to induce plasma membrane depolarization, thus preventing VOCCs from opening.

Purinergic receptors are classified into types P1 and P2, responsive to adenosine and ATP_e respectively. ATP_e is degraded to AMP and adenosine by an ecto-ATPase present at high level on the plasma membrane of most cell types. Sertoli cells express high levels of this ATP_e-degrading activity [13] and it is con-

ceivable that ATP_e effects could be due to stimulation of P1 receptors, known to be activated in Sertoli cells by ATP_e degradation products [11,12]. However, the observation that ATP_e is a potent agonist of P2-purinergic receptors and that the non-hydrolysable analogue ATP[S] is also a stimulant suggests that ATP_e hydrolysis is not necessary for its action and P1-purinergic receptors are not involved. On the other hand there is experimental evidence that AMP and adenosine do not induce any effect on $[Ca^{2+}]_i$ in rat Sertoli cells [13]. To date there are no conclusive data regarding the identification of the P2-purinergic receptor subtype(s) expressed on rat Sertoli cell plasma membrane. P2Z-receptors, present on mast cells, macrophages and fibroblasts, can be excluded too since they are coupled to the opening of plasma membrane pores that allow the passage of solutes of low molecular mass (up to 900 Da), such as ethidium bromide [26], an effect never observed under our experimental conditions at any ATP_e tested dose (results not shown). Sertoli cells clearly express G-protein-coupled P2-receptors. Structural evidence suggests that these receptors, whether activated by ATP or UTP, can all be grouped within the same family of seven membrane-spanning receptors. At present we do not know whether Sertoli cells express two separate binding sites; however, the observation that 2-methylthioadenosine-5'-triphosphate (2MeSATP), an activator of the typical P2Y-purinergic receptor, is inactive suggests that Sertoli cells might express a member of the P2Y-receptor family. Receptors that respond to ATP, UTP and ATP[S] but not to 2MeSATP have been previously described [27].

The presence of P2X receptors in rat Sertoli cells was recently excluded on the basis of inability of P2X-receptor agonists to trigger PI turnover [13]. We think that this is not convincing evidence since: (1) P2X receptors are coupled to activation of a channel that allows the permeation of Na⁺ (and other univalent cations) and Ca²⁺ and not to PI breakdown; (2) in our experimental conditions we showed that ATP_e stimulates Na⁺-dependent plasma membrane depolarization, a response usually linked to P2X-receptor activation. Yet, the putative P2X receptor expressed by Sertoli cells shows a pharmacological profile distinct from that of more typical P2X receptors, but similar to the P2X receptor recently cloned from PC12 cells [28], as a well known agonist such as AMP-CPP is inactive. Therefore we cannot exclude that ATP_e activates in Sertoli cells a single class of P2 receptor linked to both plasma membrane depolarization and Ca²⁺ release from stores. Similar sensitivity of these two responses to pertussis toxin inhibition would favour this hypothesis. However, preliminary experiments show that oxidized ATP, a selective inhibitor of ionotropic P2 receptor, inhibits depolarization without affecting Ca²⁺ release from stores. This would suggest expression of two different receptors. The in-

complete inhibition of cellular responses by pertussis toxin treatment is in agreement with indirect evidence, demonstrating that ATP_e inhibition of the effects of FSH on Sertoli cells were only partially blocked by pertussis toxin treatment [13]. Finally it is possible that the different ionic events evoked by ATP_e in rat Sertoli cells could be triggered independently by receptor activation via interaction with distinct G-proteins, one of which is sensitive to pertussis toxin (G_i/G_o family) while the other is not (G_q/G₁₁ family).

The physiological role of ATP_e and P2-purinergic receptors in the modulation of Sertoli cell functions is a matter of debate. Extracellular ATP_e mediates a variety of effects on many different cell types [1] and also in the testis this nucleotide can exert a regulatory role as demonstrated previously [10,13]. The problem raises the questions of if, and which, cells produce and release ATP_e near Sertoli cells. ATP_e is released during neurotransmitter exocytosis at cholinergic and adrenergic synapses [1,29–31] and has been proposed to exert an important role in the modulation of granulosa cell functions in the ovary [32]. Within the testis are present adrenergic and cholinergic nerves [33] and it is possible that ATP_e released from these nerve endings modulates Sertoli cell functions. Furthermore some cell types present in the seminiferous tubule could be capable of releasing ATP_e in the extracellular space. Although the presence of ATP_e in the seminiferous tubular fluid has not yet been reported, our results suggest a possible role for ATP_e as one of the agents that physiologically regulate Sertoli cell functions (for example, inducing the secretion of autocrine, paracrine and endocrine factors, thus allowing communication between adjacent Sertoli and germ cells). The recent demonstration that germ cells *in vitro* activate the PI pathway in rat Sertoli cells, thus inhibiting the response of these cells to FSH [34], together with the experimental evidence that ATP_e inhibits the FSH-dependent effects on Sertoli cells [13], supports the hypothesis that ATP_e, putatively released by germ cells, could be responsible for the modulation of Sertoli cell functions.

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