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Extracellular ATP stimulates estradiol secretion in rat Sertoli cells in vitro: modulation by external sodium

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Abstract

In this study, we examined the effects of extracellular ATP (ATPe) on $[Ca^{2+}]_i$, $[Na^+]_i$, plasma membrane potential changes and estradiol secretion in rat Sertoli cells. ATPe caused a rapid rise of $[Ca^{2+}]_i$ with an initial spike followed by a long lasting plateau. The first rapid spike was dependent on the release of Ca^{2+} from internal stores as it also occurred in Ca^{2+}-free medium while the long lasting plateau phase was dependent on Ca^{2+} influx from the external medium. ATPe stimulated a rapid plasma membrane depolarization that was dependent on an influx of Na^+ from the external medium as demonstrated by plasma membrane potential monitoring in Na^+-free medium and by $[Na^+]_i$ measurement with the Na^+-sensitive fluorescent dye SBFI. ATPe stimulated estradiol secretion in a dose dependent manner and was fully dependent on the presence of Na^+ in the external medium while the presence of Ca^{2+} was not necessary. Among the different nucleotides tested, only ATP, ATP-5′-[γ-thio]triphosphate, UTP, α,β-methylene-ATP were effective in stimulating estradiol secretion. These results demonstrate that rat Sertoli cells possess P2-purinergic receptors belonging to the P2X and P2Y subfamily which activation induces $[Ca^{2+}]_i$ and $[Na^+]_i$ rise and Na^+-dependent plasma membrane depolarization leading to estradiol secretion.

Keywords: ATP; Purinergic receptors; Sertoli; Calcium; Sodium; Estradiol

1. Introduction

In recent years, an increasing number of studies has been performed demonstrating the role of different nucleotides as extracellular mediators of important biological responses in many different cell types (Burnstock, 1990; Dubyak and El-Moatassim, 1993). In particular, it has been demonstrated that extracellular ATP (ATPe) modulates contractility of smooth muscle cells (Satchell, 1990) and cardiac miocytes (Olsson and Pearson, 1990), modulates the function of immune system cells (Di Virgilio et al., 1989), acts as a neurotransmitter in the central nervous system (Edwards et al., 1992) and plays an important role in the exocrine and endocrine system (Li et al., 1991; Soltoff et al., 1992). Recently, it has become evident the role of this nucleotide within the testis as ATPe regulates important functions in testicular cells via the activation of specific P2-purinergic receptors located on the plasma membrane of human sperm and rat Sertoli and Leydig cells (Foresta et al., 1995, 1996b). These receptors are classified in different subtypes namely P2X, coupled to the opening of ion channels, P2Y, coupled via G-protein, to internal Ca^{2+} stores depletion and P2Z, now known as P2X7, coupled to pore formation on cell plasma membrane permeable to solutes of Mw up to 900 kDa (MacKenzie et al., 1999). In rat Sertoli cells, we have recently performed functional and pharmacological studies demonstrating the existence of P2X and P2Y purinergic receptors which activation stimulates an influx of Na^+ from the external medium determining plasma membrane depolarization and a release of Ca^{2+} from internal stores (Foresta et al., 1995). Little is known on the possible biological role of this nucleotide and P2-purinergic receptors activation in Sertoli cells.

In this study, we have examined the effects of ATPe on estradiol secretion in rat Sertoli cells.

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2. Materials and methods

Sertoli cell isolation and culture: Testes were isolated from 18-day-old Sprague–Dawley rats and placed in DMEM. After decapsulation, testes were digested utilizing a modification of the procedure developed by Gorczynska and Handelsman (1991). Briefly, the testes were placed in DMEM containing trypsin (2.5 g/l), 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 preparations contain about 95% Sertoli cells as identified by light microscopy. The accuracy of Sertoli cell isolation was determined by the ability of FSH to increase $[Ca^{2+}]_i$ as previously demonstrated (Gorczynska and Handelsman, 1991) (not shown). Sertoli cells were plated on Petri dishes and 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% O_2 and 5% CO_2. Medium was replaced on every day and on day 3 of culture, contaminating germ cells were lysed by treatment with hypotonic solution for 3 min in 20 mM Tris–HCl at pH 7.5 as described by Galdieri et al. (1984). After washing cells for three times with DMEM and 24 h of recovery, cells monolayers were utilized for experiments. Sertoli cells in culture were stimulated with increasing doses of ATP (1.0, 10, 100 μM, 1.0 and 2.5 mM) in the presence of 100 nM testosterone in the bathing medium. In the evaluation of the effects of other nucleotides on estradiol secretion, only one concentration was tested (100 μM). Twenty four hours later media were collected and saved for estradiol measurement. At the end of each experiment Sertoli cell viability was evaluated by trypan blue exclusion test. Under our experimental conditions cell viability was always higher than 95% except for incubation with high ATP concentrations (2.5 mM) that lead to a decrease of cell viability. For this reason, the maximal ATP concentration utilized in the present study was 1.0 mM. In some experiments Sertoli cells were incubated in Ca^{2+}-free (no Ca^{2+} added and 0.1 mM EGTA) or Na^+-free (*N*-methyl-D-glucamine substituted) medium.

Measurement of $[Ca^{2+}]_i$: $[Ca^{2+}]_i$ was measured utilizing the fluorescent probe fura-2/AM (Foresta et al., 1992). Sertoli cells isolated as above were incubated for 30 min at 37°C in the presence of fura-2/AM (2 μM). After loading, Sertoli cells were washed free of the dye by centrifugation at 300 × *g* for 10 min, resuspended in saline and maintained at room temperature until used. $[Ca^{2+}]_i$ was measured in a LS50B Perkin–Elmer fluorometer equipped with a thermostatted and magnetically stirred cuvette holder and utilizing 1.0 ml Sertoli cells aliquots containing 7.5×10^5cells. The excitation wavelength was alternated between 350 and 380 nm and emission fluorescence was continuously monitored at 505 nm.

Evaluation of Sertoli cells plasma membrane potential changes: Plasma membrane potential changes were monitored utilizing the potential sensitive fluorescent dye bis-oxonol as previously described (Foresta et al., 1992). Briefly, 7.5×10^5 Sertoli cells were placed in a cuvette thermostatted at 37°C containing the bis-oxonol solution (200 nM) in saline. After stabilization of the fluorescent signal addition of ATP was made. Excitation and emission wavelengths were 540 and 580 nm, respectively.

Measurement of $[Na^+]_i$ in Sertoli cells suspensions: Intracellular free Na^+ was evaluated utilizing the fluorescent sodium-binding dye benzofuran isophtalate acetomethylester (SBFI/AM), as previously described (Foresta et al., 1996a). Sertoli cells, suspended in standard saline, were incubated with 5 μM SBFI/AM in the presence of the non-ionic detergent pluronic acid (20% in dimethylsulfoxide, 1:1 to SBFI/AM) for 60 min at 37°C in continuos stirring. Cells were then washed by centrifugation (two times at 250 × *g* for 10 min at room temperature) in standard saline. After centrifugation, the supernatant was discarded, cells resuspended in standard saline and kept at room temperature until used. All experiments were performed within 90 min of the dye loading. SBFI fluorescence was monitored at the wavelength pair 345 and 490 nm for excitation and emission, respectively.

Evaluation of Sertoli cell plasma membrane permeability: Sertoli cell plasma membrane permeability was monitored by measuring ethidium bromide uptake, as previously described (Murgia et al., 1993). Sertoli cell suspensions 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.

Estradiol measurement: Estradiol was measured by RIA method according to the method of Escobar et al. (1976) using a specific antibody cross-reacting less than 1% with estriol and estrone. Intra- and inter-assay coefficients of variation were 7.5% and 9.8%, respectively.

Statistical analysis: Experimental data were analysed using the Stat View II (Abacus Concepts, Berkeley, CA) statistical package. Statistical analysis was carried out using analysis of variance (ANOVA) and Student's "*t*-test". A "*p*" value of less than 0.05 was chosen as the limit for statistical significance.

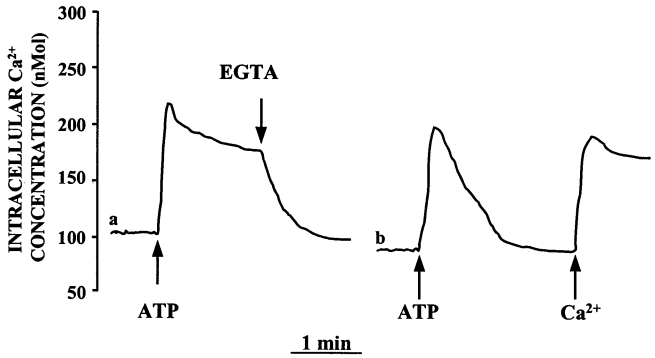

Fig. 1. Effects of ATPe on $[Ca^{2+}]_i$ in Sertoli cells: Sertoli cells (7.5×10^5 cells/ml) were loaded with fura-2/AM as described in Section 2 Traces are representative from five similar experiments for ATPe-induced $[Ca^{2+}]_i$ rise in Ca^{2+}-containing (trace a) and Ca^{2+}-free (trace b) medium. Where indicated ATP (100 μM) and Ca^{2+} (1.8 mM) were added.

3. Results

Effects of ATPe on $[Ca^{2+}]_i$: In the presence of extracellular Ca^{2+}, ATPe induced a rapid rise of $[Ca^{2+}]_i$ that was characterized by two components (Fig. 1): a first transient peak followed by a second long lasting plateau that was dependent on an influx of Ca^{2+} from the external medium as demonstrated by its inhibition by chelation of external Ca^{2+} with EGTA. The first rapid phase of the increase in $[Ca^{2+}]_i$ was due to release of intracellular Ca^{2+} since it also occurred in the absence of external Ca^{2+} (Fig. 1). When Ca^{2+} was added back to the Ca^{2+}-free medium, the second sustained plateau phase described in Ca^{2+}-containing medium was completely restored.

Evaluation of plasma membrane potential variations: The monitoring of Sertoli cell plasma membrane demonstrates that ATPe stimulates a fast and long lasting depolarization (Fig. 2, trace a) that was completely independent by the presence of Ca^{2+} in the external medium (Fig. 2, trace b) and completely dependent on the presence of Na^+ in the external medium since in Na^+-free, *N*-methyl-D-glucamine substituted medium the ATPe-dependent plasma membrane depolarization was not detectable (Fig. 2, trace c).

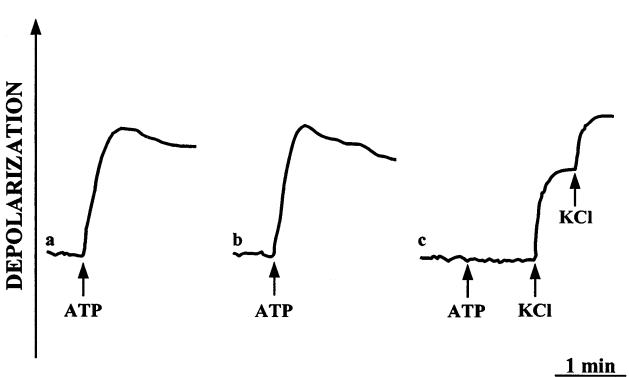

Fig. 2. Effects of ATPe on plasma membrane potential in Sertoli cells: Sertoli cells (7.5×10^5 cells/ml) were incubated in the presence of 200 nM bis-oxonol in control (trace a), Ca^{2+}-free (trace b) and Na^+-free (trace c) saline. Where indicated ATP (100 μM) and KCl (30 mM) were added. Depolarization is expressed as arbitrary units of fluorescence increase. Traces are representative of five similar experiments.

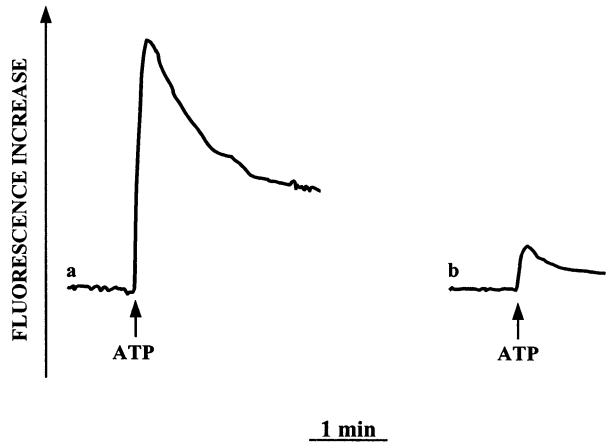

Fig. 3. Effects of ATPe on $[Na^+]_i$ in Sertoli cells: Sertoli cells (7.5×10^5 cells/ml) were loaded with SBFI/AM as described in Section 2. After loading, Sertoli cells were suspended in normal Na^+-containing (125 mM, trace a) and low Na^+-containing medium (10 mM, trace b). Traces are representative of five similar experiments for ATPe-induced $[Ca^{2+}]_i$ rise in Ca^{2+}-containing (trace a) and Ca^{2+}-free (trace b) medium. Where indicated ATP (100 μM) was added.

Effects of ATPe on $[Na^+]_I$: Given the dependency on extracellular Na^+ of plasma membrane depolarization induced by ATPe, we evaluated $[Na^+]_i$ utilizing the Na^+-sensitive fluorescent dye SBFI. As shown in Fig. 3, ATPe stimulated a rapid rise of $[Na^+]_i$ that was greatly reduced when Na^+ concentration was lowered in the extracellular medium.

Effects of ATPe on plasma membrane permeability: Sertoli cell plasma membrane permeability was evaluated measuring ethidium bromide uptake after ATPe addition. As shown in Fig. 4, this nucleotide did not induce any increase in ethidium bromide uptake while Sertoli cell permeabilization by digitonin rapidly induced the uptake of this dye.

Effects of ATPe on estradiol production: Fig. 5 shows that ATPe stimulates estradiol secretion in a dose-dependent manner with minimal and maximal efficacious dose at 1 and 100 μM, respectively. Higher ATPe doses did not increase significantly estradiol secretion. The stimulatory effects of ATPe on estradiol production was observed also in the absence of Ca^{2+} in the external medium thus excluding a role for this ion in ATPe effects. In Na^+-free medium (*N*-methyl-D-glucamine substituted medium), ATPe stimulation of estradiol was greatly reduced while 8-BrAMP was still able to stimulate estradiol secretion (not shown).

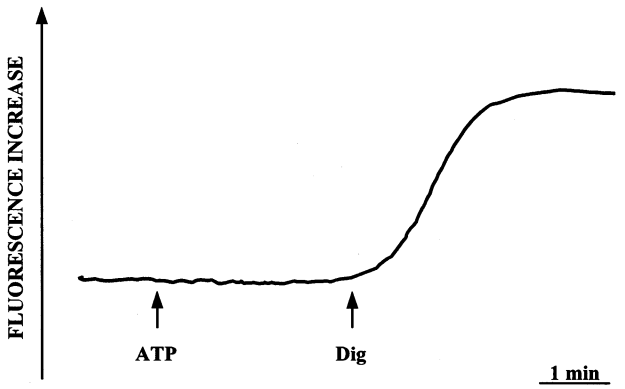

Fig. 4. Effects of ATPe on plasma membrane permeability in Sertoli cells: Sertoli cells (7.5×10^5 cells/ml) were incubated in the presence of ethidium bromide (20 μM) as described in Section 2. Trace is a representative experiment from three similar. Where indicated ATP (100 μM) and digitonin (5 μg/ml) were added.

Previous studies have shown that pertussis toxin inhibits ATPe induced rise of $[Ca^{2+}]_i$ and plasma membrane depolarization. In Fig. 6, it is shown that pre-treatment of Sertoli cells with pertussis toxin induced a significant reduction of ATPe stimulated estradiol secretion.

Effects of other nucleotides on estradiol production in rat Sertoli cells: In Table 1 are reported the effects of different nucleotides on estradiol secretion in rat Sertoli cells. Beside ATP, only its non-hydrolizable analogue ATP-5′-[γ-thio]triphosphate and UTP stimulated Sertoli cell estradiol production at micromolar concentrations and to an extent similar to that induced by ATPe.

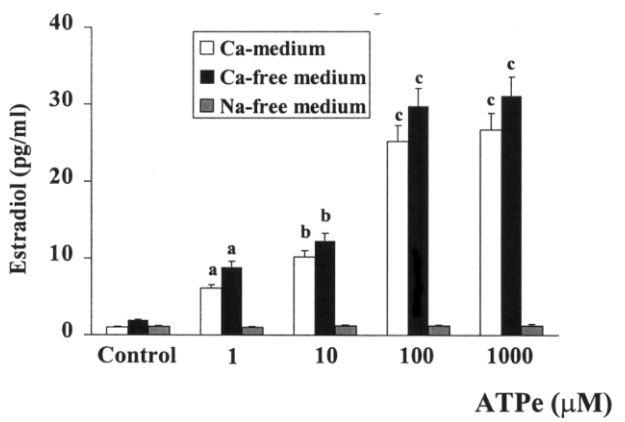

Fig. 5. Effects of ATPe on estradiol production in Sertoli cells: cells were cultured for 4 days in control medium. On the fourth day in culture, cells were stimulated with ATPe (1, 10, 100 and 1000 μM). After 24 h, media were collected and estradiol production determined by radioimmunoassay. For evaluation of ATPe-induced estradiol secretion, Sertoli cells were incubated in different experimental conditions as reported in figure legend. Values are expressed as mean ± S.D. of three separate experiments performed in duplicate: $a = p < 0.05$; $b = p < 0.01$; $c = p < 0.001$ vs control and Na^+-free medium.

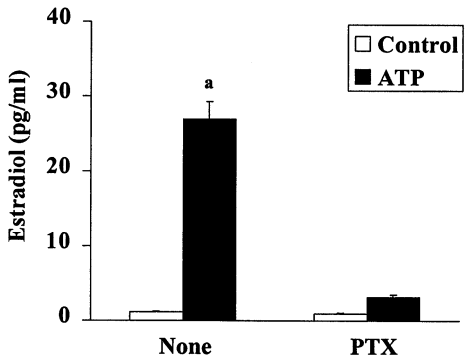

Fig. 6. Pertussis-toxin inhibits estradiol production induced by ATPe in rat Sertoli cells. Cells were cultured for 3 days in control medium. On the fourth day in culture, cells were pre-incubated in the absence or presence of pertussis toxin (PTX, 500 ng/ml for 5 hours) before addition of ATPe (100 μM). After 24 h, media were collected and estradiol production determined by radioimmunoassay. Values are expressed as mean + S.D. of three separate experiments performed in duplicate. $a = p < 0.01$ vs control and PTX treated Sertoli cells.

Also, α,β-Me-ATP was effective in stimulating estradiol secretion although to a lesser extent.

4. Discussion

The role of nucleotides as important modulators of different cell functions in many tissues is now well established (Burnstock, 1990; Dubyak and El-Moatassim, 1993; Ralevic and Burnstock, 1998). Within the testis, there is a complex network of cell–cell interactions co-ordinating the activities of the different testicular somatic and germinal cells and ATPe seems to play a primary role as an extracellular regulator of these cell–cell interactions (Foresta et al., 1995; Lalevée et al., 1999). Previous studies demonstrated that ATPe

Table 1
Effects of different nucleotides on estradiol secretion in rat Sertoli cells[a]

Nucleotide	% ATP effect
ATP	100
ATP[S]	98.6
UTP	93.4
ADP	1.1
Adenosine	0
AMP	0
GTP	0
CTP	0
α,β-Me-ATP	28.9
β,γ-Me-ATP	0
2-Me-S-ATP	0

[a] For estradiol secretion evaluation, Sertoli cells were incubated with different nucleotides as described in Section 2. The effects on estradiol production of 100 μM nucleotides are expressed as percentage of maximal estradiol secretion induced by 100 μM ATP. Values given are averages of three separate experiments.

stimulates $[Ca^{2+}]_i$ through the activation of phosphoinositide breakdown and internal Ca^{2+} stores and an influx of Ca^{2+} from the external medium (Filippini et al., 1994; Foresta et al., 1995). The results of the present study show additional evidence on the role of ATPe in the regulation of Sertoli cell functions demonstrating that this nucleotide stimulates estradiol production in a dose dependent manner and completely independent from the presence of Ca^{2+} in the bathing medium. The lack of any inhibitory effect of Ca^{2+} removal from the external medium on estradiol secretion in rat Sertoli cells has been previously demonstrated also for FSH (Talbot et al., 1991). It has been demonstrated that in rat Sertoli cells ATPe induces a plasma membrane depolarization dependent on an influx of Na^+ from the external medium since it was completely absent in Na^+-free medium. The secretion of estradiol induced by ATPe was also related to the presence of Na^+ in the bathing medium since in Na^+ -free conditions ATPe-induced effects on estradiol secretion were inhibited while the cAMP analogue 8-BrAMP was still able to stimulate estradiol secretion to demonstrate that in these experimental conditions Sertoli cells are fully competent in the secretion of estradiol. Furthermore, in agreement with the involvement of pertussis toxin-sensitive G-protein in the ATPe induced effects on $[Ca^{2+}]_i$ and plasma membrane depolarization, Sertoli cells incubation with pertussis toxin reduced the effects of ATPe on estradiol production. These results demonstrate that Sertoli cells possess specific P2-purinergic receptors for ATPe on their plasma membrane whose activation leads to $[Ca^{2+}]_i$ rise, Na^+ influx and plasma membrane depolarization followed by stimulation of estradiol production. Purinergic receptors are classified into two types, P1 and P2, responsive to adenosine and ATPe respectively. ATPe is metabolized to adenosine and AMP by ecto-ATPases present on the plasma membrane of Sertoli cells (Filippini et al., 1994). On these bases, it is possible that ATPe effects could be due to activation of P1 receptors by adenosine (Monaco et al., 1984, 1988), but since ADP does not possess any stimulatory effect on Sertoli cells and non-hydrolizable analogues of ATP are potent agonists of Sertoli cells functions, it is possible to affirm that ATPe degradation is not necessary for its action and then that P1-purinergic receptors are not involved in ATPe action in Sertoli cells. P2 purinergic receptors are classified in two major classes: P2X and P2Y (MacKenzie et al., 1999). P2X receptors are ligand-gated ion channels while P2Y are members of the family of seven membrane-spanning receptors coupled to intracellular Ca^{2+} store depletion via G-proteins. The presence of these receptors in Sertoli cells has been previously reported (Filippini et al., 1994; Foresta et al., 1995).

P2X receptors are widely expressed in many mammalian cells and are divided into seven subtypes, namely P2X1 through P2X7, coupled to the opening of plasma membrane ion channels permeable to Ca^{2+} and univalent cations as Na^+ and K^+. P2X7 receptors resemble closely in their properties the P2Z purinergic receptors (MacKenzie et al., 1999; Ralevic and Burnstock, 1998) coupled to the opening of membrane pores on plasma membrane permeable to solute with a Mw up to 900 kDa, an effect never detected in rat Sertoli cells thus excluding the presence of P2X7 in these cells. Pharmacological evidence suggest that P2X2, P2X4 and P2X5 may be absent in Sertoli cells as these cells were responsive, although to a lesser extent with respect to ATPe, to α,β-methylene-ATP, an hallmark of the presence of P2X1 and P2X3 purinergic receptors subtype (MacKenzie et al., 1999). Further studies will be necessary to precisely identify the P2X receptor subtype(s) expressed in rat Sertoli cells. The present study confirms the presence of P2 purinergic receptors in rat Sertoli cells and demonstrate that their activation promotes estradiol secretion that seems to be completely dependent on the presence of Na^+ in the external medium. The role of extracellular Na^+ in the secretion of different hormones has been previously reported in many different endocrine cells as somatotroph (Kato and Sakuma, 1997), gonadotroph (Van Goor et al., 1996) and also in testicular cells as demonstrated for Leydig cells (Rossato et al., 1997). The present study extend these knowledges demonstrating that also in Sertoli cells extracellular Na^+ plays a primary role in estradiol secretion stimulated by ATPe. While the presence of functional P2-purinergic receptors in rat Sertoli cells is well established, the intracellular mechanisms triggered by the activation of these receptors remain to be fully elucidated. In particular it is not known the nature of the effectors involved in ATPe action other than Ca^{2+} and Na^+ ions. It has been previously demonstrated that ATPe activates phosphoinositide breakdown leading to diacylglycerol and IP_3 production, the physiological activators of protein kinase C (PKC) and intracellular Ca^{2+} release (Filippini et al., 1994). Then, it is possible that also protein kinase C (PKC) may be involved in ATPe effects on estradiol production through the phosphorylation of specific substrates. The involvement of PKC in ATPe action has been previously suggested in different cell types (Morales et al., 2000). To this respect other studies have demonstrated that PKC activity and intracellular Ca^{2+} are involved in the regulation of aromatase activity in Sertoli cells (Monaco and Conti, 1987). Other signalling pathways may be involved in ATPe effects in Sertoli cells as recently demonstrated in other cell types (Aimond et al., 2000; Weyden et al., 2000) and further studies will be necessary to clarify these aspects.

The physiological role of ATPe in the regulation of Sertoli cell functions is not completely understood. As demostrated previously, this nucleotide exerts a number of different regulatory effects in different cell types and

for Sertoli cells the questions raised regard if and from where ATPe derives to stimulate these cells. It is well known that ATPe is released during neurotransmitter release at cholinergic, adrenergic and purinergic nerve endings (Burnstock, 1972; Dubyak and El-Moatassim, 1993), and it has been previously suggested to exert an important role in the modulation of granulosa cell functions in the ovary (Morley et al., 1994). Autonomic nerve fibres have been described close to Sertoli cells and have been demonstrated to be cholinergic and adrenergic (Prince, 1992; Rauchenwald et al., 1995) thus it is possible that ATP released from these nerve endings modulates Sertoli cell functions. Furthermore, it cannot be excluded that some cell types within the seminiferous tubule can release ATP contributing to the communication between Sertoli and germ cells. The observations that ATPe inhibits the modifications of Sertoli cells induced by FSH (Filippini et al., 1994) and that germ cells in vitro stimulate the hydrolysis of phosphoinositides in rat Sertoli cells leading to inhibition of their response to FSH (Welsh and Ireland, 1992) seem to support the hypothesis that ATPe could be one of the mediators of germ-Sertoli cell communications.

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