Effects of purinergic agonists on aromatase and gamma-glutamyl transpeptidase activities and on transferrin secretion in cultured Sertoli cells

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Abstract

To study the role of extracellular nucleosides and nucleotides in the regulation of Sertoli cells, the effects of agonists which occupy A_1 and P_2 purinergic receptors on aromatase and gamma-glutamyl transpeptidase (γ -GTP) activities and on transferrin secretion were tested. Sertoli cell treatment with purinergic agonists for a prolonged period of time (72 h) resulted in an increase in aromatase activity under basal conditions. In cultures stimulated with FSH, purinergic agonists counteracted the inhibitory effect on aromatase activity that long-term treatment with FSH promoted. The effects of prolonged treatments with purinergic agonists on the other two parameters of Sertoli cell function were less pronounced. Neither γ -GTP activity nor transferrin secretion was modified under basal

conditions. On the other hand, under conditions where cell differentiation was favored by FSH treatment, reductions in γ -GTP activity and transferrin secretion were usually observed. The results obtained in dbcAMP-stimulated cultures suggested that A_1 agonists exert their regulatory function at the level of cAMP formation while P_2 agonists act at a more distal point. The fact that morphological changes induced by FSH were reversed by both types of agonists, while those induced by dbcAMP were only abrogated by P_2 agonists, supports this hypothesis. In summary, these results demonstrate that purinergic agonists may be important in the regulation of Sertoli cell function.

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Introduction

The role of nucleosides and nucleotides in metabolism is well established but their potential importance as extracellular chemical messengers has been recognized only recently (Burnstock 1990). They are now known to participate in a variety of biological responses (Fradkin *et al.* 1982, Martin *et al.* 1985, Shima 1986, Hopwood & Burnstock 1987, Billig & Rosberg 1988, Loubatieres-Mariani & Chapal 1988). These responses are mediated by specific surface purinergic receptors: P_1 receptors, which have high affinity for extracellular adenosine and AMP, and P_2 receptors with high affinity for ADP and ATP (Paton & Taerum 1990).

P_1 receptors have been subclassified into A_1 , A_{2A} , A_{2B} and A_3 receptors on the basis of differing agonist affinity and on the different effects on adenylate cyclase (Londos & Wolff 1977, Stehle *et al.* 1992, Carruthers & Fozard 1993). Subclassification of P_2 receptors includes five different subtypes at the moment and the biochemical signals they elicit are more variable (Dubyak 1991, Barnard *et al.* 1994).

In Sertoli cells, Monaco *et al.* (1988) have studied the biochemical properties of the P_1 purinergic receptors and the biological effects of adenosine. Those authors have shown that P_1 receptors present in these cells belong to A_1 subtype. In agreement with what has been shown for other tissues, they are coupled to a G_i protein and their occupancy leads to inhibition of adenylate cyclase. In accordance with this inhibition, several parameters of Sertoli cell function – follicle-stimulating hormone (FSH)-dependent secretion of pyruvate (Conti *et al.* 1989), FSH stimulation of aromatase activity (Monaco *et al.* 1984) and FSH stimulation of inhibin secretion (Conti *et al.* 1988) – are negatively modulated by adenosine receptor agonists.

Histochemical studies showed that A_1 receptors are concentrated at the interface between Sertoli and germinal cells (Monaco & Conti 1986). It has been suggested that adenosine receptors on Sertoli cells are part of a paracrine system in which adenosine can be released when changes in the metabolic status in germinal cells occur (Conti *et al.* 1989).

As for P_2 receptors a recent study has shown that Sertoli cells possess the P_{2U} subtype which is associated with

phosphatidylinositol turnover and intracellular Ca^{2+} mobilization. Occupancy of these receptors causes inhibition of the FSH-dependent increase in intracellular cAMP and of the cAMP-mediated cell shape changes (Filippini *et al.* 1994). A more recent report (Foresta *et al.* 1995) showed that ATP action is also coupled to selective Na^+ influx and the authors postulated that membrane depolarization causes opening of voltage-gated Ca^{2+} channels that contribute to the sustained Ca^{2+} response. As yet, the physiological role of P_2 purinergic receptors in the regulation of Sertoli cell function remains to be clearly determined.

In the present study we have investigated whether prolonged treatment of Sertoli cell cultures with purinergic agonists regulates aromatase and gamma-glutamyl transpeptidase (γ -GTP) activities and transferrin secretion under basal and FSH-stimulated conditions.

Materials and Methods

Materials

Ovine FSH (NIH-oFSH-S-16) was obtained from the National Hormone and Pituitary Program, NIDDK (National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD, USA). Tissue culture media were purchased from Grand Island Biological Co. (Grand Island, NY, USA). All other drugs and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Sertoli cell isolation and culture

Sertoli cells from 20-day-old Sprague–Dawley rats were isolated as previously described (Schteingart *et al.* 1989). Briefly, decapsulated testes were digested with 0.1% collagenase and 0.06% soybean trypsin inhibitor in Hanks' balanced salt solution for 5 min at room temperature. Seminiferous tubules were saved, cut and submitted to 1 M glycine–2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 min at room temperature to remove germinal cells. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium which consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 20 mM Hepes, 100 IU/ml penicillin, 2.5 $\mu\text{g}/\text{ml}$ amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 $\mu\text{g}/\text{ml}$ transferrin, 5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{g}/\text{ml}$ vitamin E and 4 ng/ml hydrocortisone. Sertoli cells were cultured in 24-well plates (10 μg DNA/well) at 34 °C in a mixture of 5% CO_2 :95% air. Cells were allowed to attach for 48 h. Treatments started on day 3 and were performed in culture medium without the addition of insulin.

Purity of Sertoli cells reached 95% after 5 days in culture as seen by phase contrast microscopy (see Fig. 5A). No myoid cell contamination was revealed when an immunoperoxidase technique was applied to Sertoli cell cultures using a specific antiserum to alpha-smooth muscle actin. Remaining cell contaminants were of germ cell origin.

Culture conditions

(–) N^6 -(R-phenylisopropyl)-adenosine (PIA, 100 nM) and N^6 -cyclohexyl-adenosine (CHA, 100 nM) were used as agonists for A_1 receptors. ADP (100 μM) and ATP (100 μM) were used to elicit responses through P_2 receptors. Concentrations of purinergic agonists were chosen in accordance with the literature (Monaco *et al.* 1988, Filippini *et al.* 1994) and also with our personal data (Tables 1 and 3).

For short-term treatment, cells were maintained under basal conditions until the 6th day, when purinergic agonists were added with the aromatization mixture. Twenty-four hours later, media were collected and saved for estradiol determinations.

For long-term treatment, to evaluate the effects of purinergic agonists on γ -GTP activity and on transferrin secretion, purinergic agonists were added every day for 4 days beginning on day 3. FSH or dbcAMP stimulation was performed on day 3 and on day 6 when medium was changed. γ -GTP activity was determined on the cells harvested on day 7. The 72-h conditioned media obtained on day 6 were used to evaluate transferrin levels. In parallel cultures, cells which had received three pulses of purinergic agonists (days 3, 4 and 5) were washed and aromatase activity was evaluated in the absence of purinergic agonists.

Cell viability at the end of the culture period was evaluated by a Trypan Blue exclusion test. Under all experimental conditions tested cell viability was higher than 95%.

Aromatase assay

To evaluate aromatase activity, 100 ng/ml FSH and 3 μM testosterone as substrate for aromatization were added on the 6th day with the medium change. Media were collected 24 h later (day 7), centrifuged at 1100 g for 10 min and stored at –20 °C until estradiol determinations were performed. The monolayers were washed with 0.9% NaCl solution and 0.5 ml 2 mM EDTA (pH 7.4) was added. Cells were disrupted by ultrasonic irradiation and adequate aliquots for DNA determinations were saved.

γ -Glutamyl transpeptidase assay

γ -GTP activity was assayed by the method of Orlowsky & Meister (1963) using L- γ -glutamyl p-nitroanilide as

substrate and glycylglycine as the acceptor molecule. Sertoli cell monolayers were disrupted by ultrasonic irradiation in 0.5 ml reaction buffer (0.1 M Tris buffer, 0.01 M MgCl₂, 0.02 M glycylglycine, pH 9.0). Adequate aliquots for DNA determinations were saved and 5 mM substrate (L- γ -glutamyl p-nitroanilide) was added to the remaining material. The reaction was allowed to proceed for 120 min at 34 °C, and the enzymatic reaction was stopped by addition of acetic acid to 1 M concentration. Samples were then centrifuged and absorbances determined in a spectrophotometer at 410 nm. Values were compared against a standard curve with increasing concentrations of p-nitroaniline.

Transferrin determination

Rat transferrin was measured by RIA as described by Handelsman *et al.* (1989). A polyclonal antibody raised against rat transferrin in rabbits was used (Cappel Laboratories, Cochranville, PA, USA). The cross-reactivity of human transferrin in this assay is less than 0.003%. Unconditioned medium containing 10 μ g/ml human transferrin did not show any cross-reactivity in this assay. This RIA has a sensitivity of 3 ng/tube and intra- and interassay coefficients of variation of 7 and 16% respectively.

Other assays

DNA was determined by the method of Labarca & Paigen (1980). RIA of estradiol was performed according to the method of Escobar *et al.* (1976), using a specific antibody which cross-reacts less than 1% with estrone and estriol. This RIA has a sensitivity of 6.25 pg/tube and intra- and interassay coefficients of variation of 8 and 15% respectively.

Statistical analysis

Statistical analysis was performed by ANOVA followed by Tukey's protected *t*-test for comparison of data from multiple groups using the GB-STAT version 4.0 statistical program (Dynamic Microsystems, Inc., Silver Spring, MD, USA).

Results

Effect of purinergic agonists on FSH- and dbcAMP-stimulated estradiol production in short-term treatments

In order to evaluate the possible role of purinergic agonists on the FSH-stimulated aromatase activity, this enzyme activity was measured in the presence and absence of purinergic agonists. Initial experiments were performed to determine the appropriate doses of CHA and ATP which

Table 1 Effect of CHA and ATP on FSH-stimulated estradiol production. Values are mean $\pm$ S.D. of triplicate incubations in one representative experiment out of three

Estradiol production (pg/ μ g DNA)	
Treatment	
Control	74.9 $\pm$ 3.6
CHA	
1 nM	67.4 $\pm$ 6.5
10 nM	54.2 $\pm$ 4.8*
100 nM	49.7 $\pm$ 4.5*
1000 nM	52.2 $\pm$ 3.9*
ATP	
1 μ M	69.7 $\pm$ 9.0
10 μ M	60.7 $\pm$ 4.2*
100 μ M	53.8 $\pm$ 3.2*
1000 μ M	48.8 $\pm$ 3.2*

Cells were cultured for 5 days in control medium. On the 6th day cells were stimulated with FSH (100 ng/ml) and testosterone (3 μ M) without purinergic agonists (control) or with the addition of variable doses of CHA or ATP. Twenty-four hours later media were collected and estradiol production determined by RIA.

*P < 0.05 vs control.

Figure 1 Effect of purinergic agonists on FSH-stimulated estradiol production. Cells were cultured for 5 days in control medium. On the 6th day in culture, cells were stimulated with FSH (100 ng/ml) and testosterone (3 μ M) without purinergic agonists (control) or with the addition of PIA (100 nM), CHA (100 nM), ADP (100 μ M) or ATP (100 μ M). Twenty-four hours later media were collected and estradiol production determined by RIA. Values are expressed as mean $\pm$ S.D. of triplicate incubations in one representative experiment out of three. Different superscripts indicate statistically significant differences (*P* < 0.05).

elicited a response on estradiol production (Table 1). The lowest doses of CHA and ATP which produced a statistically significant inhibition of estradiol production were 10 nM and 10 μ M respectively. Figure 1 shows that not only 100 nM CHA but also 100 nM PIA (both A₁ receptor agonists) inhibited FSH-stimulated estradiol

Table 2 Effect of purinergic agonists on dbcAMP-stimulated estradiol production. Values are expressed as mean ± s.d. of triplicate incubations in one representative experiment out of three

Treatment	Estradiol production (pg/μg DNA)
Control	40.9 ± 1.2 ^a
PIA	40.4 ± 3.7 ^a
CHA	44.2 ± 0.2 ^a
ADP	23.5 ± 0.9 ^b
ATP	28.0 ± 4.0 ^b

Cells were cultured for 5 days in control medium. On the 6th day cells were stimulated with dbcAMP (0.2 mM) and testosterone (3 μM) without purinergic agonists (control) or with the addition of PIA (100 nM), CHA (100 nM), ADP (100 μM), or ATP (100 μM). Twenty-four hours later media were collected and estradiol production determined by RIA. Different superscripts indicate statistically significant differences ($P<0.05$).

production as previously shown by other authors (Monaco *et al.* 1984). Figure 1 also shows that inhibitory results similar to those obtained with 100 μM ATP were obtained with 100 μM ADP (both P_2 receptor agonists).

Table 2 shows the effect of purinergic agonists on dbcAMP-stimulated aromatase activity. Under these experimental conditions, ADP and ATP inhibited estradiol production while PIA and CHA had no effect.

Effect of purinergic agonists on aromatase activity in long-term treatments

In this set of experiments, stimulation with purinergic agonists was performed for 3 days and the agonists were removed before aromatase activity was determined. Again, initial experiments to determine the appropriate doses of CHA and ATP were performed (Table 3). The lowest doses of CHA and ATP which produced a statistically significant stimulation of aromatase activity were 10 nM and 10 μM respectively. Figure 2 shows that under basal conditions prolonged treatment (72 h) with 100 nM CHA and PIA, and 100 μM ATP and ADP produced similar stimulatory effects. Figure 2 also shows that prolonged treatment with FSH decreased aromatase activity, while a combination of FSH with purinergic agonists resulted in an aromatase activity which was similar to that obtained in basal control cultures.

Table 4 shows that prolonged treatment with dbcAMP also resulted in inhibition of aromatase activity and that this inhibition was also observed when dbcAMP was combined with CHA. On the other hand, when dbcAMP was combined with ATP, the dbcAMP inhibitory effect was not observed.

Effect of purinergic agonists on γ-GTP activity

To study the possible involvement of purinergic agonists in the regulation of basal and FSH-stimulated γ-GTP

Table 3 Effect of prolonged treatment with CHA and ATP on aromatase activity. Values are mean ± s.d. of triplicate incubations in one representative experiment out of three

Treatment	Estradiol production (pg/μg DNA)
Control	43.9 ± 3.6
CHA	
1 nM	44.1 ± 5.0
10 nM	64.2 ± 3.8*
100 nM	75.1 ± 6.2*
1000 nM	81.4 ± 2.3*
ATP	
1 μM	44.1 ± 5.1
10 μM	60.1 ± 6.8*
100 μM	74.9 ± 5.2*
1000 μM	62.0 ± 7.8*

Cells were maintained for 3 days without additions (control) or with the daily addition of variable doses of CHA or ATP. On the 6th day pretreated monolayers were washed twice and FSH (100 ng/ml) and testosterone (3 μM) were added. Twenty-four hours later media were collected and estradiol production determined by RIA.

* $P<0.05$ vs control.

Figure 2 Effect of prolonged treatment with purinergic agonists on basal and FSH-regulated aromatase activity. Cells were maintained for 3 days under basal (open bars) or FSH-stimulated (100 ng/ml, hatched bars) culture conditions without purinergic agonists (control) or with the daily addition of PIA (100 nM), CHA (100 nM), ADP (100 μM) or ATP (100 μM). On the 6th day, pretreated monolayers were washed twice and FSH (100 ng/ml) and testosterone (3 μM) were added. Twenty-four hours later media were collected and estradiol production determined by RIA. Values are expressed as mean ± s.d. of triplicate incubations in one representative experiment out of three. Different superscripts indicate statistically significant differences ($P<0.05$).

activity, we added these agonists to basal and FSH-stimulated cultures daily for a 4 day period. Figure 3 shows that prolonged treatment with PIA, CHA, ADP or ATP did not modify basal γ-GTP activity. On the other hand,

Table 4 Effect of prolonged treatment with purinergic agonists on dbcAMP-regulated aromatase activity. Values are mean $\pm$ S.D. of triplicate incubations in one representative experiment out of three

Treatment	Estradiol production (pg/ μ g DNA)
Basal	53.2 $\pm$ 3.9 ^a
dbcAMP	34.6 $\pm$ 6.1 ^b
dbcAMP+CHA	30.9 $\pm$ 1.9 ^b
dbcAMP+ATP	59.1 $\pm$ 2.6 ^a

Cells were maintained for 3 days under basal or dbcAMP-stimulated (0.2 mM) culture conditions with the daily addition of CHA (100 nM) or ATP (100 μ M). On the 6th day pretreated monolayers were washed twice and FSH (100 ng/ml) and testosterone (3 μ M) were added. Twenty-four hours later media were collected and estradiol production determined by RIA.

Different superscripts indicate statistically significant differences ($P < 0.05$).

Figure 3 Effect of prolonged treatment with purinergic agonists on basal and FSH-stimulated γ -GTP activity. Cells were maintained for 4 days under basal (open bars) or FSH-stimulated (100 ng/ml, hatched bars) culture conditions without purinergic agonists (control) or with the daily addition of PIA (100 nM), CHA (100 nM), ADP (100 μ M) or ATP (100 μ M). γ -GTP activity was evaluated on the 7th day. Values are expressed as mean $\pm$ S.D. of triplicate incubations in one representative experiment out of three. Different superscripts indicate statistically significant differences ($P < 0.05$).

ADP and ATP inhibited FSH-stimulated γ -GTP activity while PIA and CHA had no effect.

Table 5 shows that similar results were obtained when the FSH stimulus was replaced by dbcAMP.

Effect of purinergic agonists on transferrin secretion

Figure 4 shows that treatment with PIA, CHA, ADP or ATP for 72 h did not modify basal transferrin secretion while all treatments inhibited FSH-stimulated transferrin secretion.

Table 5 Effect of prolonged treatment with purinergic agonists on dbcAMP-regulated γ -GTP activity. Values are mean $\pm$ S.D. of triplicate incubations in one representative experiment out of three

Treatment	γ -GTP activity (pmol/ μ g DNA/min)
Basal	16.1 $\pm$ 3.0 ^a
dbcAMP	39.5 $\pm$ 2.7 ^b
dbcAMP+CHA	36.6 $\pm$ 3.2 ^b
dbcAMP+ATP	29.2 $\pm$ 1.7 ^c

Cells were maintained for 4 days under basal or dbcAMP-stimulated (0.2 mM) culture conditions with the daily addition of CHA (100 nM) or ATP (100 μ M). γ -GTP activity was evaluated on the 7th day.

Different superscripts indicate statistically significant differences ($P < 0.05$).

Figure 4 Effect of prolonged treatment with purinergic agonists on basal and FSH-stimulated transferrin secretion. Cells were maintained for 3 days under basal (open bars) or FSH-stimulated (100 ng/ml, hatched bars) culture conditions without purinergic agonists (control) or with the daily addition of PIA (100 nM), CHA (100 nM), ADP (100 μ M) or ATP (100 μ M). The 72-h accumulated medium was collected and transferrin concentration was determined by RIA. Values are expressed as mean $\pm$ S.D. of triplicate incubations in one representative experiment out of three. Different superscripts indicate statistically significant differences ($P < 0.05$).

Table 6 shows that CHA did not modify dbcAMP-stimulated transferrin secretion. It also shows that ATP inhibited dbcAMP-stimulated transferrin secretion.

Effects of different treatments on Sertoli cell morphology

Figure 5A shows Sertoli cells in culture forming a confluent epithelioid monolayer. Figures 5B and C show that no changes in cell morphology were observed in cultures stimulated with CHA and ATP respectively. Figures 5D and G show the characteristic morphological changes provoked by FSH and dbcAMP respectively. Co-treatment with CHA reversed the morphological

Table 6 Effects of prolonged treatments with purinergic agonists on dbcAMP-regulated transferrin secretion. Values are mean $\pm$ S.D. of triplicate incubations in one representative experiment out of three

Treatment	Transferrin (ng/ μ g DNA)
Basal	25.2 $\pm$ 4.2 ^a
dbcAMP	83.3 $\pm$ 10.2 ^b
dbcAMP+CHA	79.8 $\pm$ 1.6 ^b
dbcAMP+ATP	58.6 $\pm$ 9.3 ^c

Cells were maintained for 3 days under basal or dbcAMP-stimulated (0.2 mM) culture conditions with the daily addition of CHA (100 nM) or ATP (100 μ M). Transferrin was determined by RIA in the 72-h conditioned medium. Different superscripts indicate statistically significant differences ($P < 0.05$).

changes induced by FSH (Fig. 5E) but did not modify those promoted by dbcAMP (Fig. 5H). Finally, ATP was able to reverse the morphological changes promoted by both FSH (Fig. 5H) and dbcAMP (Fig. 5I). Pictures were taken on the 6th day after a 72-h treatment with FSH, dbcAMP and the different purinergic agonists.

Discussion

It is now well established that extracellular nucleosides and nucleotides are important regulators of cell function in several tissues (Burnstock 1990). Data reported so far on the regulation of Sertoli cell function were reviewed in the Introduction. In the present study, additional evidence of the potential role of nucleosides and nucleotides in the regulation of Sertoli cell function is presented. This evidence has been obtained by evaluating the effect of purinergic agonists on three well-known Sertoli cell parameters: aromatase and γ -GTP activities and transferrin secretion.

Sertoli cell aromatase activity is stimulated after a short-term exposure to FSH. This FSH-stimulated aromatase activity decreases markedly throughout pubertal development (Dorrington & Armstrong 1979). In 10- to 20-day-old Sertoli cell cultures it has also been shown that FSH-stimulated aromatase activity can be regulated by germ cell derived factors (Le Magueresse & Jégou 1986) and by several growth factors (Mallea *et al.* 1986, Khan & Nieschlag 1991, Morera *et al.* 1992). On the other hand, we have recently shown that prolonged treatment (96 h) with FSH provokes a decrease in the ability of FSH or dbcAMP to stimulate aromatase activity acutely. We have interpreted these results as a reflection of a more differentiated state of the cell promoted by hormonal stimulation (Schteingart *et al.* 1995).

Monaco *et al.* (1984) had previously shown that adenosine exerts a regulatory effect on FSH-dependent

estradiol production. In agreement with that report, our results show that A₁ agonists inhibit FSH- but not dbcAMP-stimulated estradiol production in short-term experiments. These results are consistent with the nature of A₁ receptors in Sertoli cells which are coupled to a G_i protein (Monaco *et al.* 1988). In addition, the results presented herein show that not only A₁ agonists but ADP and ATP, which occupy P₂ receptors, inhibit estradiol production in short-term experiments.

ADP and ATP are metabolized to AMP and adenosine in reactions catalyzed by an ecto-ATPase and a 5'-nucleotidase. These enzymes are present at the plasma membrane in different cells (Emmelot & Bos 1966, Filippini *et al.* 1990). If ATP and ADP were metabolized, their effects could be related to the action of metabolic products through A₁ receptors. Yet, two lines of evidence defy this possibility. First, the ecto-ATPase, which has been recently identified in Sertoli cells, is inactive with ADP (Barbacci *et al.* 1996). Secondly, ADP and ATP also inhibit dbcAMP-stimulated estradiol production while PIA and CHA do not.

It has been recently demonstrated that occupancy of Sertoli cell P_{2U} receptors promotes phosphoinositide turnover with the subsequent elevation of inositol triphosphate and a rapid increase in intracellular Ca²⁺ accompanied by decreased cAMP response to FSH stimulation (Filippini *et al.* 1994). This decreased cAMP response could follow a Ca²⁺-dependent activation of a cyclic nucleotide phosphodiesterase provoked by increased intracellular Ca²⁺ levels. The activation of protein kinase C (PKC) activity which follows elevations of diacylglycerol upon hydrolysis of phosphatidylinositol can also contribute to the observed phenomenon. The decrease in cAMP provoked by activation of these signal molecules might explain the inhibition of FSH-stimulated estradiol production. However, this possibility is questioned by the results mentioned before, which demonstrate the inability of dbcAMP to overcome the inhibitory effects of ATP. In addition, these results show that ATP effects are produced at a site distal to cAMP production. Our results agree with previous observations demonstrating that PKC activity and intracellular Ca²⁺ levels are involved in the regulation of aromatase activity at a site distal to cAMP accumulation (Monaco & Conti 1987, Talbot *et al.* 1991). ATP regulation of other steroid-producing cells, like granulosa and Leydig cells, has recently been presented (Kamada *et al.* 1994, Foresta *et al.* 1996).

The effects produced by occupancy of purinergic receptors described so far in this paper, and those published by other authors, have been obtained in short-term (24 h) stimulation periods and little is known about the consequences that prolonged treatments may have on Sertoli cell function. We have observed that a 72-h treatment with A₁ and P₂ agonists under basal conditions results in an increase in aromatase activity. Conti *et al.* (1989) had previously observed a similar effect in

Figure 5 Micrographs of Sertoli cells. (A) control cells; (B) CHA 100 nM; (C) ATP 100 μ M; (D) FSH 100 ng/ml; (E) and (F) combined treatments with 100 ng/ml FSH and CHA 100 nM or ATP 100 μ M respectively; (G) dbcAMP 0.2 mM; (H) and (I) combined treatments with 0.2 mM dbcAMP and CHA 100 nM or ATP 100 μ M respectively. All treatments lasted 72 h. The selected microscopic fields are representative of predominant morphology of the cells in triplicate incubations that were repeated three times. Horizontal bar (in (A)) represents 20 μ m.

long-term experiments with A₁ agonists. Those authors considered the fact that long-term activation of a receptor could lead to desensitization. However, they observed that A₁ agonists were still able to inhibit cAMP production after long-term experiments and we have observed that purinergic agonists are still able to modulate aromatase activity after long-term treatments. Altogether, these results suggest that the observed effects are not related to desensitization but to other mechanisms which remain to be elucidated. In spite of the molecular mechanisms involved, these results suggest that cell exposure to purinergic agonists for prolonged periods of time promotes a less differentiated state of Sertoli cells. This latter proposal is further sustained by the observations made in FSH-stimulated cultures where purinergic agonist treatments have been shown to counteract the differentiating effects of FSH.

As mentioned before, we have also analyzed the effect of purinergic treatments on two other parameters of Sertoli cell function which may also be regarded as markers of Sertoli cell differentiation (Hodgen & Sherins 1973, Skinner *et al.* 1989, Chaudhary *et al.* 1996). γ -GTP activity increases with the age of the Sertoli cell donor (Lu & Steinberger 1977, Sanborn *et al.* 1986) and it has been shown to be stimulated by FSH and by germ cell secreted products (Schteingart *et al.* 1989). Transferrin can also be stimulated by FSH (Skinner & Griswold 1980), several growth factors (Skinner & Griswold 1982) and germ cell derived factors of unknown nature (Le Magueresse *et al.* 1988). To our knowledge no reports are available on the potential effect that prolonged treatments with purinergic agonists may have on these two parameters of Sertoli cell function. Our results show that under the latter experimental conditions regulation of γ -GTP activity and transferrin secretion does exist. However, the changes observed were less pronounced than those described for aromatase activity. Neither γ -GTP activity nor transferrin secretion was modified by purinergic treatments under basal conditions. These results probably reflect the fact that these parameters do not change with cell differentiation as markedly as aromatase activity does. On the other hand, under conditions where cell differentiation is favored by FSH treatment a reduction in γ -GTP activity and transferrin secretion was usually observed.

Similar signal transduction pathways to those mentioned for the regulation of aromatase activity by A₁ and P₂ agonists in short-term experiments are probably involved in the observed long-term regulatory effects. In all cases the regulatory effects of ATP, but not those of CHA, were also observed when FSH was replaced by dbcAMP stimulation. Similar observations were made on the morphological changes induced by these agonists. Altogether the results obtained indicate that in addition to the ability of ATP to regulate FSH stimulated cAMP levels, this nucleotide can exert regulatory actions which are produced beyond cAMP formation. As to the results obtained

with ATP on γ -GTP activity, they are in agreement with our recent observations demonstrating that FSH-stimulated γ -GTP activity can be regulated by a PKC-dependent pathway at a site distal to cAMP formation (Meroni *et al.* 1997).

It has been reported that ATP may cause cell membrane permeation and cytolysis in different cells (Filippini *et al.* 1990). We think that the effects reported here for ATP are due to specific interactions with membrane receptors rather than to nonspecific cytotoxic effects as (1) a well-preserved morphology was observed after a 72-h stimulation period with ATP, and (2) Trypan Blue exclusion tests showed that cell viability was higher than 95%.

In conclusion, these results demonstrate that Sertoli cells can be regulated by purinergic agonists. The source of these purinergic agonists in the extracellular space is still unknown but the possibility that they may represent components of the complex network involved in paracrine/autocrine actions is attractive.

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