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Lack of endogenous adenosine receptor activation explains the insensitivity of neonatal rat Leydig cells to treatment with pertussis toxin

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Abstract

We have previously shown that the G_i protein-mediated negative modulation of FSH-stimulated cAMP production in neonatal rat Sertoli cells can be blocked by pretreatment with pertussis toxin (PT), an inhibitor of the G_i protein function. In contrast, hCG-stimulated testosterone (T) production by neonatal Leydig cells was insensitive to PT action, despite the demonstration of G_i protein in these cells by immunohistochemistry. We now tested the hypothesis that the missing G_i protein function in neonatal Leydig cells is due to absence of its endogenous activator, adenosine. It was first reconfirmed in dispersed cells of 7-day-old rats that PT preincubation enhanced FSH-stimulated cAMP production (i.e. Sertoli cell function), but had no effect on hCG-stimulated T production (Leydig cell function). The adenosine agonist *n*-phenylisopropyladenosine (PIA 1 μmol/l), acting through the G_i-protein, attenuated FSH-stimulated cAMP production, and this effect was abolished when the G_i protein was inactivated by preincubation with PT. PIA (1 μmol/l) also inhibited ($P < 0.05$–0.01) the basal and hCG-stimulated rates of Leydig cell T production, and the PIA effect was abolished by PT preincubation. In conclusion, the present data show that the apparent absence of a functional G_i protein function of neonatal Leydig cells is due to insufficient endogenous levels of the stimulating ligand, adenosine. When the G_i protein function is activated by exogenous adenosine agonist (PIA), the enhancement of hCG-stimulated T production by PT can be demonstrated. At the same time, the endogenous level of adenosine is sufficient for activation of the G_i protein function in Sertoli cells.

Keywords: Inhibitory guanine-nucleotide-binding regulatory protein; Rat testis; Testosterone; Signal transduction pathways

1. Introduction

Adenosine has long been postulated to modulate testicular function [1,2]. Four separate adenosine receptors are known; the A_1 and A_3 adenosine receptors, which inhibit adenylyl cyclase [3–6], and the A_2a and A_2b adenosine receptors, which stimulate adenylyl cyclase [7–9]. A_1 receptors are present in both tissue sections and cultured cells of the testis [2,10–12]. A_3 receptor mRNA expression is high in spermatocytes [13]. Neither Northern blot analysis nor in situ hybridization studies revealed hybridization signals for A_2a or A_2b receptor mRNA in the testis, suggesting that the stimulating adenosine receptor subtypes are not present in rat testis [14]. In contrast, the inhibitory A_1 and A_3 adenosine receptors were readily detected and found functional in the testis [14].

Sertoli cells express adenosine receptors in vitro [15] and in vivo [11]. Adenosine and the adenosine analog *n*-phenyliso-propyladenosine (PIA) inhibited FSH-stimulated cAMP production in cultured Sertoli cells of the rat [12,15,16] and hamster [17]. The inhibition was mediated via the pertussis toxin (PT) sensitive inhibitory component of the G-protein complex, i.e. the G_i protein [12]. Huhtaniemi et al. [18] reported that in rat seminiferous tubules, PT-sensitive G_i protein is present in both high-FSH-responsive (XIV–V) and low-FSH-responsive (VII–VIII) stages of the epithelial cycle, but PT treatment increased FSH-stimulated cAMP production only in the low-responsive stages VII–VIII, suggesting that in these stages of the cycle,

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spermatogenic cells inhibit FSH response via the G_i-protein mediated pathway. It was suggested that in low responsive stages, the germ cell product activating the G_i-protein-mediated pathway is either adenosine itself or a subtance increasing the seminiferous tubular adenosine content [18,19]. Kangasniemi [20] reported that there were stage-specific mechanisms for adenosine-dependent regulation of FSH-stimulated cAMP production in the rat seminiferous epithelium.

We have earlier shown [21] that, although the PT-sensitive G_i-protein function was present in the postnatal rat testis, it was specifically lacking in the Leydig cells. Nevertheless, the G_i protein was detected in neonatal Leydig cells by immunohistochemistry [22], but for unknown reasons, it appeared not to be endogenously activated. The aim of the present study was to test the hypothesis that lack of a sufficient endogenous level of adenosine is the reason why the G_i-protein-mediated negative modulation of Leydig cell function is not detected in the neonatal rat testis.

2. Materials and methods

2.1. Materials

Pertussis toxin (PT), *n*-phenylisopropyladenosine (PIA), 1-methyl-3-isobutylxanthine (MIX), DNAse, bovine serum albumin (BSA), theophylline and collagenase were obtained from Sigma Chemical Comp. (St. Louis, MO, USA). [^{125}I]-Testosterone (2000 Ci/mmol) was purchased from Orion-Farmos Diagnostica (Oulunsalo, Finland), and Na[^{125}I]iodide and [a^{32}P]-dCTP (3000 Ci/mmol) from The Radiochemical Centre (Amersham International Plc, Bucks, UK). Medium 199, glutamine and gentamycin were purchased from GIBCO (Paisley, Scotland, UK). Human chorionic gonadotropin (hCG) (CR-121; 13 500 IU/mg by bioassay) was prepared by Dr R. Canfield (Columbia University, New York, NY, USA), and provided by NICHD (NIH, Bethesda, MD, USA). Recombinant human (rec) FSH (Org 32489, approximately 10 000 IU/mg), was donated by Organon (Organon International BV, Oss, The Netherlands).

2.2. Animals

Sprague-Dawley derived male rats, aged 7 days, were used for measurement of testosterone (T) and cyclic adenosine 3′,5′-monophosphate (cAMP) production in testis cell cultures (see below). The animals were kept in constant conditions of light (14 h on, 10 h off) and temperature (21–22°C), and allowed food and water ad libitum. The animals were killed by decapitation and the testes processed as described later.

2.3. Tissue preparation and cell culture

Testis tissue was prepared as previously described by Warren [23] and Eskola et al. [21,22]. Tunica albuginea was removed aseptically, and the testes were placed in medium 199 always containing 0.1% BSA, 2 mmol/l glutamine, 13 mmol/l $NaHCO_3$, 50 mg/l gentamycin and in incubation, the media also contained 0.2 mmol/l MIX. The gonads were rinsed and placed in medium 199 containing 0.4% collagenase and DNAase (10^5 Kunitz units/l). The tissue was incubated at 37°C for 30–45 min and mechanically dispersed by aspirating through a Pasteur pipet at 10-min intervals. After the tissue had completely disintegrated, the cells were centrifuged at 200 × *g* for 5 min and the supernatant was discarded. The cells were washed twice and resuspended in medium 199. At the final dilution, cells from one testis were reconstituted in 1.1 ml medium. Aliquots of 0.4 ml of testis cells were placed in wells of a 24-well culture plate. The culture plates were placed in an incubator with an atmosphere of 5% CO_2 in air, and maintained at a temperature of 37°C. The cells were allowed to attach to the wells for 24 h.

2.4. Incubations

In the first set of experiments, the dispersed testis cells were incubated in the presence or absence of 0, 1, 10, 100 and 1000 nmol/l PIA. PIA was dissolved in ethanol (final concentration 0.045%) and appropriate ethanol controls were made. Ethanol had no effect on cAMP or T production (data not shown). The samples were incubated for 4 h. In the second set of experiments, the samples were incubated with recFSH (30 IU/l) in the presence or absence of PT (100 μg/l) for 4 h and the other samples were incubated with hCG (10 μg/l) in the presence or absence of PT (100 μg/l) for 4 h.

In the third set of experiments, the samples were incubated with recFSH (30 IU/l) or hCG (10 μg/l) in the presence or absence of PIA (0, 10, 100 and 1000 nmol/l) and a maximal stimulating dose of PT (100 μg/l). The samples were incubated for 4 h with or without PT. After incubations, the media collected were stored at −20°C for measurement of T, or diluted 1:1 with 2 mmol/l theophylline, heated at 100°C for 10 min, and subjected to cAMP radioimmunoassay.

2.5. Steroid and cAMP measurement

The T concentrations of the culture/incubation media were determined directly by a radioimmunoassay (RIA) as previously described [24]. The assay had an intra-assay coefficient of variation (CV) less than 6%, and the interassay CV less than 12%. cAMP was measured by RIA, using the acetylation method described by Harper

and Brooker [25]. The tracer, disuccinyl-cAMP, was radioiodinated with Na[^{125}I]-iodide [26]. This assay had intra- and interassay CVs of less than 10%.

2.6. Statistical analysis

The data were analyzed by one-way analysis of variance and Duncan's new multiple range test after logarithmic transformation. The statistical significance of the PT effects on FSH and hCG stimulation was analyzed by the Student's t-test, i.e. production in the presence versus absence of PT. A P-value less than 0.05 was chosen as the limit of statistical significance.

3. Results

3.1. Effects of PT on FSH-stimulated cAMP production and hCG-stimulated T production

The amount of cAMP produced by the testicular cells in response to FSH is presented in Fig. 1a. PT alone had no effect on cAMP production (results not shown). Cells cultured with both FSH and PT produced significantly ($P < 0.05$) more cAMP than those treated with FSH alone.

Fig. 1b shows the effects of hCG and hCG plus PT on T production by testicular cells; there was no significant difference between effects of these two groups on T production. PT alone had no effect on testicular T production (results not shown).

3.2. Effects of the adenosine analog PIA and PT on basal and FSH-stimulated cAMP production

PIA alone had no effect on testicular basal cAMP production at any concentration studied (Fig. 2a). However, it clearly suppressed the FSH-stimulated cAMP production at the highest concentration tested (1 μmol/l, $P < 0.01$) (Fig. 2b). In contrast, the PIA inhibition of cAMP accumulation was greatly reduced or totally abolished in cells treated with PT at all the concentrations studied (Fig. 2c).

3.3. Effects of the adenosine analog PIA and PT on basal and hCG-stimulated T production

Concentrations of 1, 10 and 100 nmol/l of PIA had no effect, but the 1 μmol/l concentration significantly inhibited basal T production ($P < 0.05$) of the rat testis cells (Fig. 3a). After 4-h incubations with hCG, T production (2.1 $\pm$ 0.2 ng/testis) was also inhibited by 1 μmol/l PIA significantly ($P < 0.01$), but not with the lower 10 and 100 nmol/l doses used (Fig. 3b). Incubation of the testicular cells with hCG increased T production (3.3 $\pm$ 0.8 ng/testis) (about 10-fold), but hCG plus PIA (1 μmol/l) decreased T production (2.1 $\pm$ 0.2 ng/testis) (Fig. 3b). However, the PIA inhibition of T production was abolished when the cells were incubated with PT (2.7 $\pm$ 0.2 ng/testis) (Fig. 3c).

4. Discussion

The present data show that there are specific mechanisms for adenosine-dependent regulation of hCG-stimulated T production and FSH-stimulated cAMP production in the 7-day-old rat testicular cells. Thus, there are adenosine receptors in gonadotropin-responsive cells of the neonatal rat testis, both in Leydig and Sertoli cells. Stimulation by gonadotropins is specific to these two cell types, since LH/hCG receptors are present only in Leydig cells [27] and FSH receptors only in Sertoli cells [28].

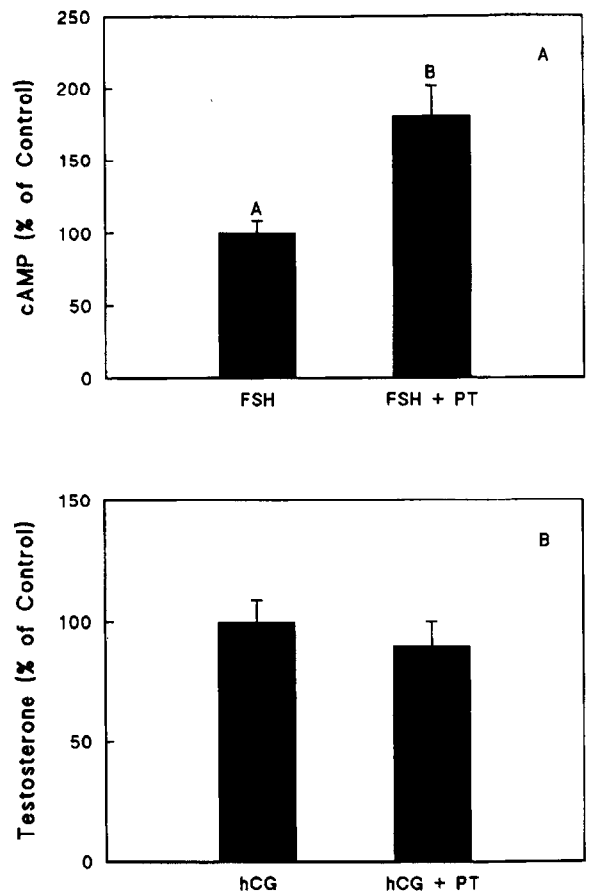


Fig. 1. cAMP production (A) and testosterone production (B) by testis cells from 7-day-old rat after 4 h in culture at 37°C. The cells were incubated with recFSH (30 IU/l) and FSH plus PT (100 μg/l) (A) or hCG (10 ng/ml) and hCG plus PT (B), and cAMP and testosterone production were assayed. The data are mean $\pm$ S.E.M. of three to four independent experiments, with quintuplicate determinations. The data indicated by bars labeled with different letters differ significantly ($P < 0.05$).

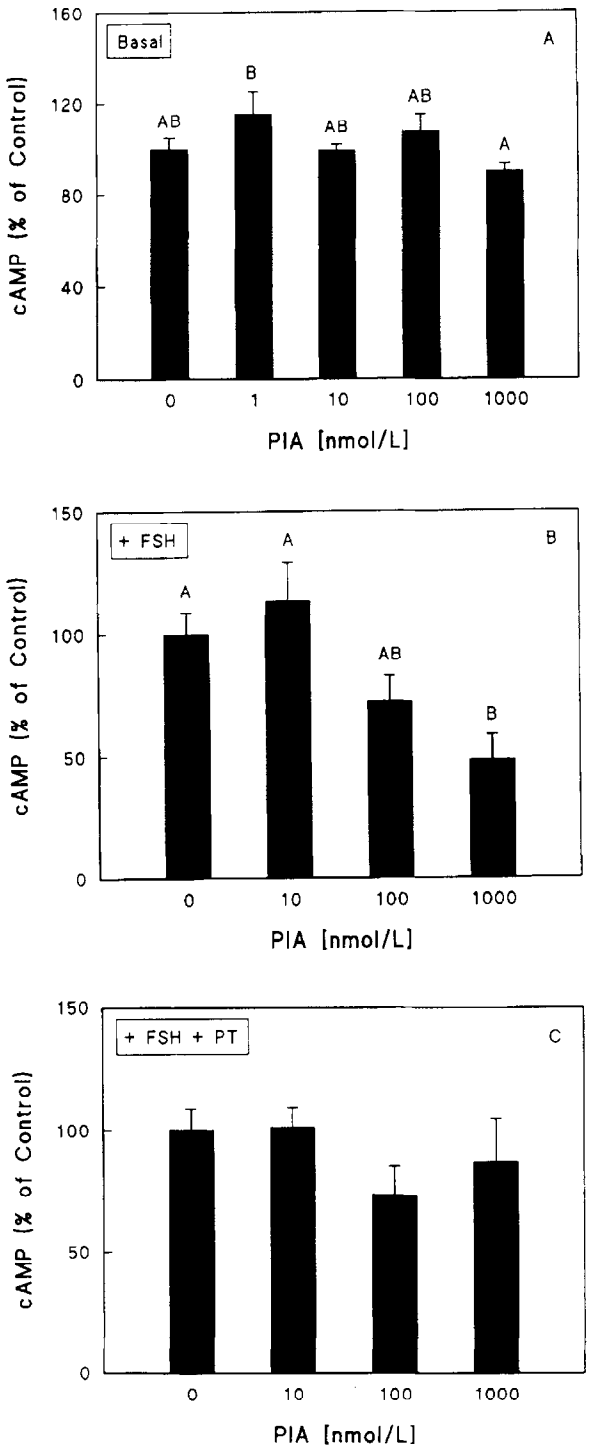


Fig. 2. Dose-responses of the cultured dispersed testicular cells from 7-day-old rats to PIA alone (A), or to PIA + FSH (30 IU/l) (B) or to PIA + FSH + PT (100 μg/l) (C). The cells were incubated for 4 h at 37°C, and their formation of cAMP was assayed. The data are mean ± S.E.M. of triplicate determinations. The data indicated by bars labeled with different letters differ significantly ($P < 0.05$).

The Sertoli cell expresses A_1-type inhibitory adenosine receptors [11]. Occupancy of these receptors has been shown to inhibit the FSH-stimulated cAMP accumulation and androgen aromatization [15], as was confirmed in the present study. These observations have led to the hypothesis that cAMP levels in these cells are

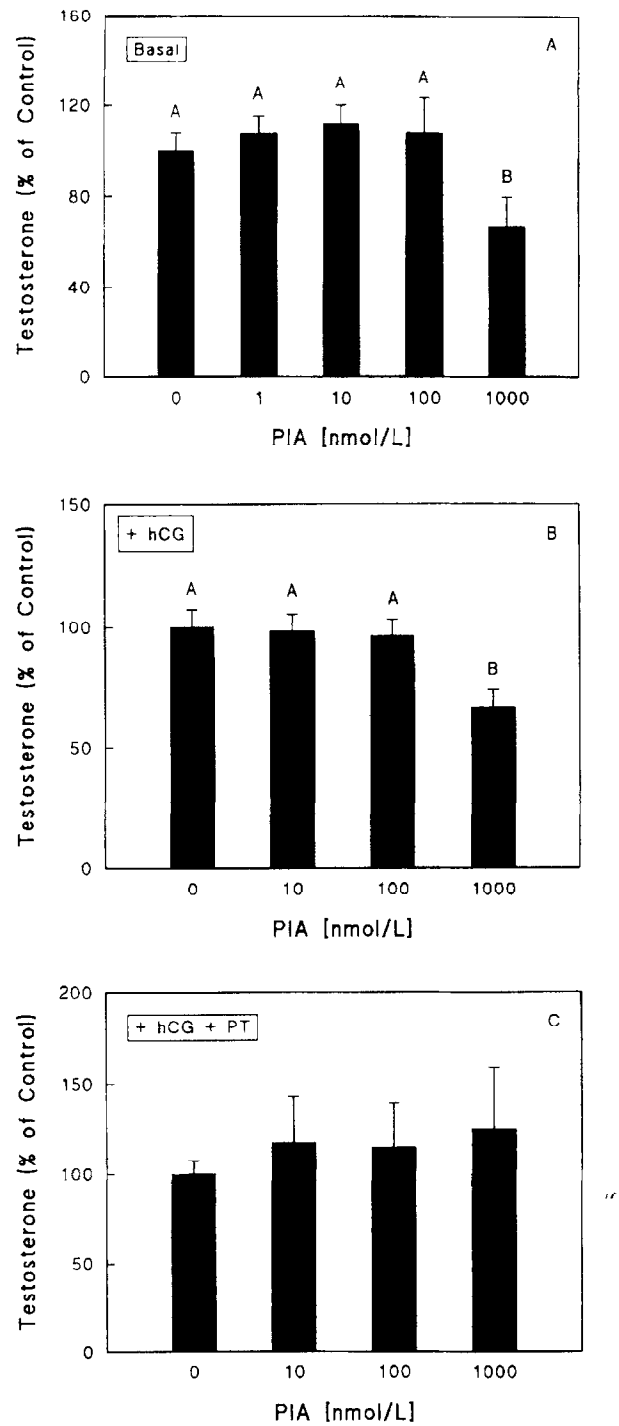


Fig. 3. Testosterone production by testis cells from rats aged 7 days to PIA alone (A), or to PIA + hCG (10 μg/l) (B) or to PIA + hCG + PT (100 μg/l) (C). The cells were incubated for 4 h at 37°C and formation of T in testicular cells was assayed. For further details, see the legend of Fig. 2.

regulated by both stimulatory and inhibitory signals. Also in Sertoli cell membrane preparations, adenosine analogs inhibit adenylate cyclase, suggesting that modulation of the cyclic nucleotide-dependent pathway is the mechanism by which adenosine regulates the function of these cells [29]. Monaco et al. [12] have demonstrated that the inhibitory adenosine A_1 receptors are coupled to the inhibitory component (G_i) of adenylate cyclase. In the Sertoli cell, inhibitory and stimulatory signals thus interact in the bimodal regulation of adenylate cyclase and intracellular cAMP.

The known adenosine receptor subtypes that stimulate adenylate cyclase are not present in the rat testis or are present at very low levels [14]. In contrast, the A_1 and A_3 receptors were both functional in the testis. Previous studies of cultured rat testis cells showed that the A_1 receptors are largely present in Sertoli cells [2,11,12,14]. In contrast, A_3 receptor mRNA was highest in germ cells and absent in Sertoli cells [13,14]. Low-level A_3 mRNA labeling was also seen on Leydig cells [14].

We have earlier shown [21] that PT pretreatment enhances CT-stimulated cAMP production of testicular cells from 5-day-old rats, thus suggesting that PT-sensitive G_i-protein function is present in the postnatal testis. We also showed that the G_i protein becomes functional, i.e. PT-sensitive, in rat Leydig cells between 8 and 15 days of age, concomitantly with testicular descent [22], and is active from that period through maturity. Here, we showed that the G_i-protein activity could be demonstrated in Leydig cells of 7-day-old rats, when they were cultured with PIA, a potent agonist for the inhibitory A_1-adenosine receptors. This indicates that there are functional adenosine receptors already in the neonatal testis, including the Leydig cells, and that the G_i protein becomes functional if the apparently low endogenous levels of adenosine are supplemented with exogenous nucleoside. This was clearly evidenced by the finding that the adenosine analog (PIA) inhibited hCG-stimulated T production in rat Leydig cells, and that the inhibition was abolished when G_i-protein function was abolished by preincubation with PT. The fact that PIA alone had a slight inhibitory effect on basal T production suggests that the shortage of endogenous adenosine is not absolute. Monaco et al. [12] reported earlier that similar adenosine and adenosine analog effects on FSH-stimulated cAMP production were seen in Sertoli cells from 15-day-old rats.

Only a few studies have focused on the development of adenosine receptors. A_1 receptors are detectable around birth and adult-like levels can be observed by one month of age, with increased number of binding sites, of similar affinity, during development in the rat brain tissue [30]. As early as at birth, these receptors are functionally linked to G-proteins. However, the potency of GTP analogs to displace cyclohexyladenosine (CHA) binding seems to be weaker in very young animals. It is suggested that there are functional changes during postnatal development in the signal transduction pathways modulated by adenosine in the brain tissue [31]. The same phenomenon may prevail in the testis tissue and play a role in regulating G_i protein function in fetal rat Leydig cells. As all cells release adenosine into the local environment, it is likely that A_1 and A_3 receptors are activated by local adenosine [32]. As has been proposed for other tissues, A_1 and A_3 receptor activation by endogenous adenosine may, in turn, act to down-regulate cell activity [32]. For metabolically active tissues, such as the testes, this may be especially important and may play a role in regulating PT-sensitive G_i protein function.

In conclusion, the present data show that there are specific mechanisms for adenosine-dependent modulation of gonadotropin-dependent T and cAMP production in the neonatal rat testis, both in Leydig cells and in Sertoli cells. The inhibition was mediated via a PT-sensitive G_i-protein, indicating that G_i-protein becomes functional through action of local adenosine, resulting in the regulation of testicular cell function. Our findings show that the endogenous adenosine concentration is sufficient to activate the G_i-protein-mediated regulatory system in postnatal rat Sertoli cells. In contrast, the endogenous adenosine concentration is apparently too low for G_i-protein activation in the neonatal Leydig cells, which explains the insensitivity of these cells to PT, despite their expression of the G_i protein, as shown previously by immunohistochemistry. The present results suggest that adenosine could play a role of a paracrine regulator in the testis and that its physiologic effects change during the ontogeny.

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