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Modulation of Gonadotropic and Purinergic Responsiveness by Islet Activating Protein in Sertoli Cells from Immature Rats

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In the present study we have examined the effects of islet activating protein (IAP) on the regulatory effects of FSH, glucagon and (−)N^6-(*R*)-phenyl-isopropyladenosine (PIA), an adenosine A_1 receptor agonist, on the formation of cAMP and estradiol-17β (E_2) in Sertoli cell cultures isolated from immature (19-day-old) rats. FSH (NIH-FSH-S-15) (1.25 μg/ml) caused a more than 10-fold stimulation of the level of both cAMP and E_2 in the spent media from Sertoli cell cultures during an 18 h incubation. Both responses were reduced by 80% in the presence of PIA (10^{-6} M). When the cultures were preincubated for 24 h with increasing concentration of IAP, the inhibitory effects of PIA were counteracted in a concentration-dependent manner. Moreover, preincubation with IAP (>20 ng/ml) caused a significant stimulation of FSH-stimulated cAMP production even in the absence of PIA. PIA inhibited FSH-stimulated cAMP production in a concentration dependent manner. However, when the cells were preincubated with IAP (100 ng/ml) for 24 h, the inhibitory effects of PIA were completely abolished, and PIA now actually caused a slight stimulation of cAMP production. Both FSH and glucagon stimulated cAMP production in a concentration-dependent manner. Preincubation with IAP (100 ng/ml for 24 h) resulted in an increase in maximal stimulation of cAMP production for both FSH and glucagon. When adenylyl cyclase (AC) activity was measured directly in isolated membrane particles from Sertoli cells cultured in the presence of IAP (100 ng/ml) for 24 h, both basal and FSH-stimulated AC activity were significantly higher than in membrane particles from control cells. These results provide a further characterization of the functional G_i component coupled to the AC complex in cultured rat Sertoli cells, mediating the inhibitory effects of adenosine and possibly other endogenous substances on cAMP production.

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INTRODUCTION

Adenosine influences adenylyl cyclase (AC) activity via at least two apparently distinct receptor subtypes [1]. At A_1 receptors adenosine inhibits AC activity via activation of the inhibitory guanine nucleotide binding protein (G_i), while at A_2 receptors it stimulates AC activity via the stimulatory guanine nucleotide binding protein (G_s).

Binding of FSH to receptors on Sertoli cells leads to stimulation of AC activity and increased production of cAMP which in turn stimulates production of other Sertoli cell products such as lactate, and estradiol-17β (E_2) [2, 3]. In addition to FSH, it has been shown that other agents, such as glucagon also stimulate cAMP production in cultured Sertoli cells [4, 5]. It has also been demonstrated that nanomolar concentrations of adenosine and adenosine-agonist inhibits cAMP production in Sertoli cells, and that this effect may be mediated via specific A_1 receptors for adenosine [6, 7].

Islet activating protein (IAP), a fraction of the pertussis toxin that can be isolated from the culture supernatant of *Bordetella pertussis*, specifically inhibits the function of G_i-protein mediating inhibition of AC activity by catalysing the transfer of the ADP-ribose moiety of NAD^+ to the α-subunit of G_i resulting in a complete loss of G_i functions [8].

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Abbreviations: AC, adenylyl cyclase; E_2, estradiol-17β; FCS, foetal calf serum; HBSS, Hanks' balanced salt solution; IAP, islet activating protein/pertussis toxin; MEM, minimum essential medium; PIA, (−)-N^6-(*R*)-phenyl-isopropyladenosine; NECA, N^5-ethyl-carboxamidoadenosine.

Conti and coworkers have now reported that pretreatment of rat Sertoli cells with pertussis toxin abolishes the inhibitory effects of adenosine analogues on FSH stimulated cAMP production [9, 10]. Similar effects have been observed in hamster Sertoli cells [11]. This work represents a further study of the effects of IAP on FSH as well as glucagon-stimulated and purine-attenuated formation of cAMP and E_2 in Sertoli cell cultures isolated from immature rats. We have also investigated effects of incubation of Sertoli cell cultures with IAP on the subsequent AC activity in isolated membrane particles from such cells.

MATERIALS AND METHODS

Chemicals and enzymes

Trypsin (1-250) was obtained from Difco; collagenase (type 1, C-0130), pancreatic deoxyribonuclease (925 Kunitz units/mg protein), adenosine deaminase (type 1, A-1030), cAMP, testosterone and E_2 were all obtained from Sigma Chemical Company, St Louis, MO, U.S.A. (−)-N^6-(*R*)-phenyl-isopropyladenosine (PIA) and N^5-ethyl-carboxamidoadenosine (NECA) were obtained from Boehringer-Mannheim, Germany. Hanks' balanced salt solution (HBSS), Eagles minimum essential medium (MEM) and supplements were all obtained from Gibco. [8-^{3}H]cAMP and [^{3}H]E_2 were obtained from Amersham, U.K. Ovine-FSH (NIH-FSH S-15) was obtained from NIADD, Bethesda, MD, U.S.A. Glucagon was from Novo, Copenhagen, Denmark. Islet activating (IAP) (lot no. 180) was purchased from List Biological Laboratories Inc., CA, U.S.A.

Preparation and culture of Sertoli cells

Isolation of Sertoli cell cultures from immature (19-day-old) rats was performed as described earlier [4]. The cultures were maintained in MEM containing 10% foetal calf serum (FCS) until day 3. Then the cells were washed and culture medium changed to MEM without FCS. On day 4, cells were washed and medium was exchanged with medium with or without IAP. After 24 h, the medium was removed. When AC activity was analysed, incubations were stopped and membranes were prepared as described below. Otherwise MEM was added containing testosterone (0.5 μM), and adenosine deaminase (2 μg/ml) and with addition of FSH and PIA as described in the figure legends. After another 18 h the spent medium was collected, immediately heated for 3 min at 80°C and subsequently stored at −70°C until assayed.

Measurement of cAMP

The samples of incubation medium were centrifuged for removal of cellular debris. Levels of cAMP were examined in 50–100 μl aliquots of the samples using a kit obtained from BDH chemicals supplemented with [8-^{3}H]cAMP. Details of the procedure which involves a simple direct assay implying a competitive protein binding technique, are described elsewhere [12].

Measurement of E_2

Levels of E_2 were determined in 200 μl aliquots of incubation medium by a direct radioimmunoassay [13].

Preparation of membrane particles

After incubation with or without IAP, medium was removed, the dishes were placed on melting ice and the cells were rinsed twice with ice-cold phosphate buffered saline and harvested with a cell scraper. Cells from several dishes were pooled and collected by centrifugation for 10 min at 800 *g* at 4°C. After removal of the supernatant, the cell pellets were stored in liquid nitrogen until preparation of membrane particle suspensions. This was performed as described by Jahnsen *et al.* [14]. In brief each cell pellet was resuspended in hypotonic TE buffer (25 mM Tris–HCl, 1 mM EDTA, pH 7.4) and homogenized at 0–4°C in a Dounce glass–glass homogenizer using 3 × 10 strokes with the tight fitting pestle. The homogenates were centrifuged for 30 min at 27,000 *g* at 4°C, washed and centrifuged again and resuspended in TE buffer, containing 0.1% BSA (Sigma A-4378), to the desired concentration (15–30 μg protein/ml).

AC assay

The procedure was essentially as described earlier [15]. In brief, aliquots (20 μl) of membrane particle suspensions were examined for AC activity in a final volume of 50 μl containing 1 mM ATP including 1–2 × 10^6 cpm of [^{32}P]ATP, GTP (40 μM), EDTA (1.4 mM), $MgCl_2$ (400 μM in excess of ATP and EDTA), cAMP (1 mM (including 10,000 cpm of [8-^{3}H]cAMP)), phosphocreatine (20 mM), creatine phosphokinase (200 μg/ml), and myokinase (20 μg/ml) in Tris–HCl buffer (25 mM) pH 7.4. The incubations were carried out in the presence of increasing concentrations of FSH (NIH-FSH S-15) at 35°C for 12 min. The reactions were stopped by adding 100 μl of stopping solution containing cAMP (10 mM), ATP (40 mM) and sodium dodecylsulphate (SDS) (1%) followed by mixing and immediate cooling at 0°C. Isolation of cAMP was performed by combined Dowex-50 and alumina chromatography as described by Salomon *et al.* [16] with the modifications of Birnbaumer *et al.* [17].

Protein measurements

Protein content was measured as described by Lowry *et al.* [18] using BSA as standard.

RESULTS

Figure 1 shows the effect of increasing concentration of IAP on FSH-stimulated and purine-inhibited cAMP and E_2 formation in cultured Sertoli cells.

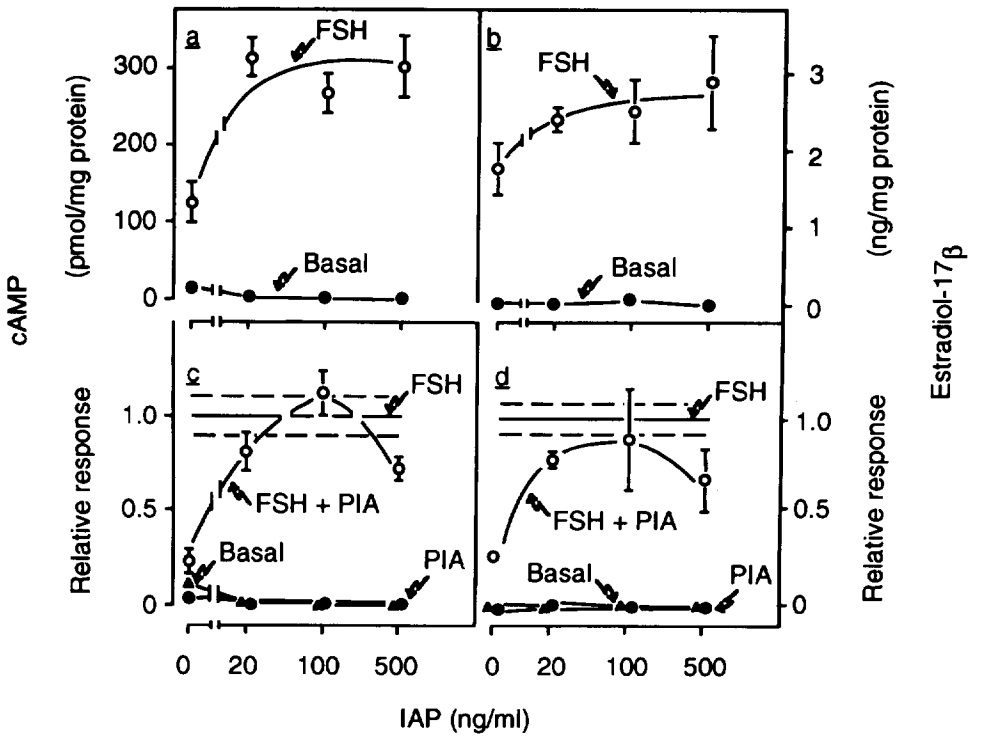

Fig. 1. Effects of increasing concentrations of islet activating protein on gonadotropic and purinergic regulation of cAMP and E_2 production in cultured rat Sertoli cells. Sertoli cell cultures from 19-day-old Sprague–Dawley rats were isolated and maintained as described in Materials and Methods. At day 4 medium was removed, and new medium containing various concentrations of islet activating protein was added. After 24 h medium was removed and new medium with or without (basal) FSH (1.25 μg/ml), (−)-N^6-(*R*)-phenyl-isopropyladenosine (PIA) (10^{-6} M), or a combination of these. Incubations were stopped after 18 h and concentrations of cAMP and E_2 were measured in the samples of incubation medium. Each point represents the mean ± SEM of 6 separate cultures.

Cultures were incubated with or without IAP in increasing concentrations for 24 h. Then medium was changed with new medium containing either a maximal stimulatory concentration of FSH (1.25 μg/ml), or a maximal inhibitory concentration of PIA (1 μM), or a combination of these. As seen in Fig. 1(a), no effects were observed on basal cAMP levels after preincubation with IAP. However, preincubation with IAP was associated with a considerable increase (2–3-fold) in FSH-stimulated cAMP production. A smaller but significant increase in FSH-stimulation of E_2 formation was also observed after preincubation with IAP [Fig. 1(b)]. Figure 1(c) shows basal and FSH stimulated cAMP production in the absence or presence of PIA. No effects of PIA were observed on basal cAMP levels. FSH caused an approx. 10-fold stimulation of cAMP production whereas the simultaneous addition of PIA reduced this effect by 80%. Preincubation with increasing concentration of IAP caused a relief from the purinergic inhibition, and a complete abolishment of the PIA effect was seen at 100 ng/ml of IAP. Similar effects of IAP were seen on aromatization. Again PIA caused a dramatic reduction of FSH-stimulated E_2 production, which was counteracted by preincubation with increasing concentration of IAP [Fig. 1(d)].

In Fig. 2 Sertoli cells were incubated with and without FSH (2.5 μg/ml) and with increasing concentration of PIA (10^{-11}–10^{-6} M) after a 24 h preincubation in the presence or absence of IAP (100 ng/ml). As seen from Fig. 2(a), PIA inhibited the FSH-stimulated cAMP production in a concentration dependent manner. However, when the cells were preincubated with IAP, the stimulatory effects of FSH was increased by more than 50%, and the inhibitory effects of PIA were completely abolished. The presence of increasing concentrations of the adenosine analogue now caused a transient increase in cAMP levels. When experiments were performed as in Fig. 2, but without addition of FSH, the levels of cAMP did not change significantly from basal. Similar observations were also made in the presence of the A_2 adenosine receptor agonist NECA (results not shown). The effects of IAP on adenosine attenuated formation of E_2 is shown in Fig. 2(b). Again, similar results were found, as preincubation with IAP (100 ng/ml) eliminated the inhibitory effects of PIA, and a transient increase in E_2 formation was observed.

Figure 3 shows the effects of preincubation with IAP (100 ng/ml) on the concentration dependent stimulation of cAMP production by FSH (20–2500 ng/ml)

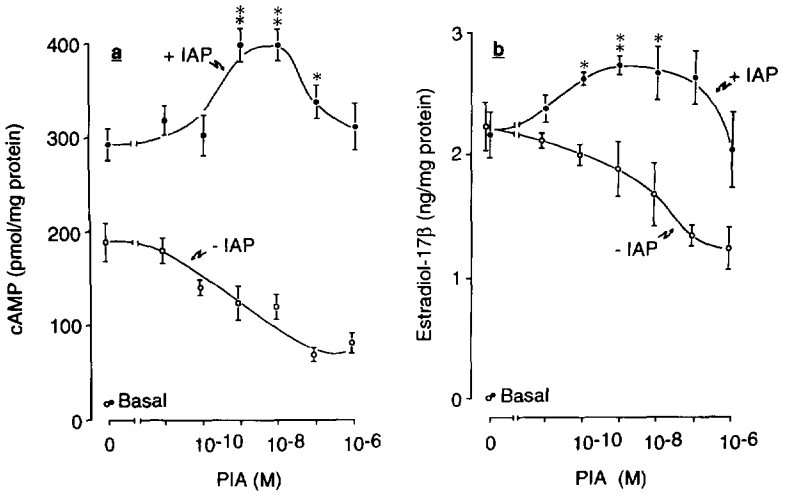

Fig. 2. Effects of increasing concentrations of $(-)$-N^6-(R)-phenyl-isopropyladenosine on FSH stimulated cAMP and E_2 production in cells preincubated with or without islet activating protein. Sertoli cell cultures from 19-day-old rats were isolated and maintained as described in Material and Methods. At day 4 medium was removed, and new medium with or without IAP (100 ng/ml) was added. After 24 h medium was removed and new medium with or without (basal) FSH (2.5 μg/ml) and PIA (10^{-11}–10^{-6} M) was added. The incubations were stopped after 18 h, and concentrations of cAMP and E_2 were measured in the samples of incubation medium. Each point represents the mean ± SEM of 6 separate cultures. Asterisks indicate significant effects of IAP pretreatment ($*0.01 < P < 0.05$; $**P < 0.01$, by Student's two sample t-test).

[Fig. 3(a)] or glucagon (40–5000 ng/ml) [Fig. 3(b)]. In the presence of hormonal concentrations which stimulated cAMP production, IAP pretreatment caused a 2-fold increase in the hormonal response for all agonist concentrations that stimulated cAMP production. Although the response in cAMP production did not completely reach a plateau level for the highest concentration of FSH investigated, the pretreatment with IAP did not have a major effect on the sensitivity (EC_{50}) of the response.

As seen from Fig. 4, addition of FSH to isolated membrane particles from Sertoli cell cultures caused a concentration dependent stimulation of AC activity. In membrane particles from cells preincubated for 24 h with IAP, the basal AC activity was almost twice the activity in membranes from non-treated cells. Further-

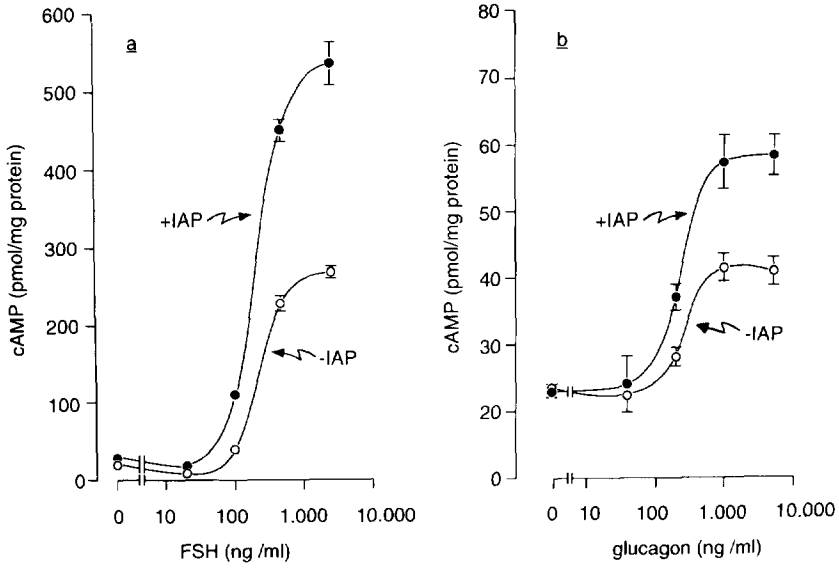

Fig. 3. Effects of increasing concentration of FSH or glucagon on cAMP production in Sertoli cells after a preincubation with or without islet activating protein. Sertoli cell cultures from 19-day-old rats were isolated and maintained as described in Materials and Methods. At day 4, medium was removed and new medium with or without islet activating protein (IAP) (100 ng/ml) was added. After 24 h, medium was removed and new medium containing increasing concentrations of either FSH (20–2500 ng/ml) or glucagon (40–5000 ng/ml) was added. After 18 h the incubation was stopped, and concentrations of cAMP in the samples of incubation medium were measured. Each point represents mean ± SEM of 6 separate cultures.

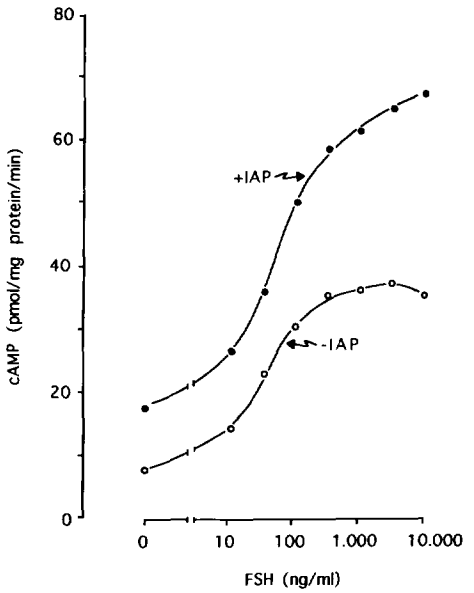

Fig. 4. Concentration dependent stimulation by FSH of adenylyl cyclase activity in membrane particles from Sertoli cells preincubated with or without islet activating protein. Sertoli cell cultures from 19-day-old rats were isolated and maintained as described in Materials and Methods. At day 4, medium was removed and new medium with or without islet activating protein (IAP) (100 ng/ml) was added. After 24 h the incubation was stopped and membrane particles were prepared from the cells and subsequently analysed for adenylyl cyclase activity as response to increasing concentrations of FSH as described in Materials and Methods. Each point represents the mean of duplicate measurements.

more, addition of FSH again caused a concentration-dependent stimulation of AC activity, and the activities in membranes from IAP treated cells were higher than those in membrane particles from control cells for all concentrations of FSH investigated.

DISCUSSION

The present study provides further evidence that A_1 receptors in rat Sertoli cells are coupled through G_i to inhibition of AC. This conclusion is based on the fact that preincubation of Sertoli cells with IAP completely counteracts the inhibitory effects of an adenosine analogue on FSH-stimulated cAMP production as well as on FSH stimulated aromatization, which is in agreement with the results in references [9–11].

IAP exerts its modulatory effects on the AC complex by catalysing ADP-ribosylation of the α-subunit ($G_{\alpha i}$) of G_i-proteins [8]. This ADP ribosylation of $G_{\alpha i}$ completely abolishes the activity of G_i and prevents the function of receptors with inhibitory effects on the AC complex.

A very interesting finding during these experiments was that after pretreatment with IAP, PIA actually caused a transient increase in cAMP levels and E_2 levels rather than a decrease. Similar observations have been made by other investigators in fat cells [19]. These authors interpreted their results as evidence that fat cells also contain A_2-receptors, which are known to stimulate cAMP production [1]. In line with this, the stimulatory effects of PIA on cAMP and E_2 formation in cells pre-treated with IAP, could indicate that cultured Sertoli cells contain A_2 receptors as well. However, the A_2 receptor agonist NECA as well as the A_1 receptor agonist PIA were unable to stimulate cAMP production in Sertoli cells even when the inhibitory effects of A_1-receptor activity on AC were blocked by pretreatment with IAP. Thus, it would appear that stimulation of cAMP production in Sertoli cells by adenosine agonists only occurs in a situation where the AC activity is simultaneously stimulated with FSH. It has recently been shown that the subtypes II and IV of the AC enzyme can be stimulated by free $\beta\gamma$-subunits of G-protein, and this effect is dependent on $G_{\alpha s}$ stimulation of the enzyme [20]. In line with this, the stimulatory effects of the adenosine analogue on cAMP production in the presence of FSH could be due to free $\beta\gamma$-subunits resulting from activation of an IAP insensitive G-protein. Alternatively, the additive stimulatory effects of FSH and PIA in IAP pretreated Sertoli cells could be due to crosstalk from A_1-receptor-mediated stimulation of another signal transduction pathway.

The present results also revealed that pretreatment of Sertoli cells with IAP was associated with a general amplification of the agonist-stimulated cAMP response from whole cells as well as in cell membranes. Similar effects have been observed in hamster Sertoli cell cultures [11]. This could indicate that G_i in Sertoli cells, under the present incubation conditions, exerts a tonic inhibitory effect on the catalytic subunit of the AC complex. One possibility is that this inhibitory effect is caused by the presence of endogenous adenosine in the cell cultures. However, these experiments were performed in the presence of high amounts of adenosine deaminase, which quickly inactivates adenosine released from the cells. In line with the effects of IAP on rat Sertoli cells, it has been reported that pretreatment with IAP is associated with a general amplification of agonist stimulated AC activity and increased cAMP production in whole cells [21–24]. In experiments on isolated rat heart cells which possess A_1 receptors for adenosine, IAP also caused a general amplification of agonist stimulated cAMP production even though the experiments were performed in the presence of adenosine deaminase [22]. Also in C^6-glioma cells preincubation with IAP revealed a similar effect in spite of the fact that these cells are not supposed to contain adenosine receptors [23]. Thus, either there must be other endogenous inhibitory agonists than adenosine or a functional G_i as such may restrict the activity of the catalytic subunit and attenuate agonist stimulated cAMP production. Other agonists than adenosine interacting with G_i-coupled

receptors in Sertoli cells from rats of this age, remain to be identified.

There are several IAP sensitive signal transducing G proteins. The G_o-protein subclass are probably mainly involved in agonist stimulation of membrane-bound phospholipase C, or activation of ion channels [see 25 for a review]. Sertoli cells contain $G_{o\alpha 2}$ in low concentrations, but the functional coupling of these G-proteins to a signal transducing system remains to be demonstrated [26]. However, it cannot be excluded that the augmentation of agonist stimulated AC activity in IAP pretreated Sertoli cells could be due to uncoupling of G_o activity.

In conclusion, these experiments show that the inhibitory effect of the adenosine A_1 receptor agonist PIA on FSH-stimulated cAMP and E_2 formation in rat Sertoli cells, is completely counteracted by preincubation with IAP. These results agree well with other reports [9–11], and provide further evidence that A_1 receptors in cultured rat Sertoli cells act on the complex via a G_i component. However, the additive effects of PIA in Sertoli cells pretreated with IAP has not previously been reported. These observations could be due to A_1-receptor activation of an IAP-insensitive signal transduction pathway in addition to AC. The fact that IAP-pretreatment also augments hormone-stimulated cAMP production broadens the putative functions of G_i in regulation of AC activity in rat Sertoli cells.

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