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Possible involvement of A_1 receptors in the inhibition of gonadotropin secretion induced by adenosine in rat hemipituitaries *in vitro*

D.L.W. Picanço-Diniz[1], M.M. Valença[2], A.L.V. Favaretto[3], S.M. McCann[4] and J. Antunes-Rodrigues[3]

[1]Departamento de Fisiologia, Centro de Ciências Biológicas, Universidade Federal do Pará, Belém, PA, Brasil
[2]Departamento de Neurologia e Psiquiatria, Centro de Ciências da Saúde, Universidade Federal de Pernambuco, Recife, PE, Brasil
[3]Departamento de Fisiologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[4]Pennington Biomedical Research Center (LSU), Baton Rouge, LA, USA

Abstract

We investigated the participation of A_1 or A_2 receptors in the gonadotrope and their role in the regulation of LH and FSH secretion in adult rat hemipituitary preparations, using adenosine analogues. A dose-dependent inhibition of LH and FSH secretion was observed after the administration of graded doses of the R-isomer of phenylisopropyladenosine (R-PIA; 1 nM, 10 nM, 100 nM, 1 µM and 10 µM). The effect of R-PIA (10 nM) was blocked by the addition of 8-cyclopentyltheophylline (CPT), a selective A_1 adenosine receptor antagonist, at the dose of 1 µM. The addition of an A_2 receptor-specific agonist, 5-N-methylcarboxamidoadenosine (MECA), at the doses of 1 nM to 1 µM had no significant effect on LH or FSH secretion, suggesting the absence of this receptor subtype in the gonadotrope. However, a sharp inhibition of the basal secretion of these gonadotropins was observed after the administration of 10 µM MECA. This effect mimicked the inhibition induced by R-PIA, supporting the hypothesis of the presence of A_1 receptors in the gonadotrope. R-PIA (1 nM to 1 µM) also inhibited the secretion of LH and FSH induced by phospholipase C (0.5 IU/ml) in a dose-dependent manner. These results suggest the presence of A_1 receptors and the absence of A_2 receptors in the gonadotrope. It is possible that the inhibition of LH and FSH secretion resulting from the activation of A_1 receptors may have occurred independently of the increase in membrane phosphoinositide synthesis.

Correspondence
J. Antunes-Rodrigues
Departamento de Fisiologia
Faculdade de Medicina de
Ribeirão Preto, USP
Av. Bandeirantes, 3900
14049-900 Ribeirão Preto, SP
Brasil

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- LH
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- A_1 receptor
- Anterior pituitary

Introduction

The characterization of adenosine receptors is currently based on the selective binding of analogues containing carboxamide groups in their structure to A_2 receptors or of purine derivatives with modifications in the N^6 position to A_1 receptors (1) and on the ability of the agonist/receptor complex to modify the activity of guanine nucleotide-binding membrane proteins (GP) (2). Consequently, type A_1 receptors were subdivided into A_{1A} receptors (which may regulate Ca^{2+} influx), A_{1C} receptors (which may regulate

K^+ efflux), and A_{1B} receptors (which may inhibit adenyl cyclase activity), with the effects of their activation possibly being mediated by membrane GP (2). More recently, coupling of the A_1 receptors to G_0 and G_i has been characterized using the R-isomer of phenylisopropyladenosine (R-PIA) as the binding agonist in the preparation (3). Ca^{2+} efflux from the cell may also be mediated by GP after the activation of A_1 receptors, a mechanism that utilizes calcium exchange with Na^+ and is dependent on a pertussis toxin-sensitive pathway (4).

New adenosine receptor subtypes have been described on the basis of their affinity for 5'-N-ethylcarboxamideadenosine (NECA). A_{2a} receptors have high affinity and A_{2b} receptors have low affinity for the agonist. Variations in tissue distribution and differences in binding capacity indicate that these receptor subtypes may be different proteins (5,6).

The activation of purinergic receptors may also modify the capacity for inositol triphosphate synthesis in different types of experimental preparations (7,8). Studies on isolated sympathetic ganglia demonstrated that endogenously released adenosine may inhibit postsynaptic stimulation and [^{3}H]myo-inositol release (8). Other data have suggested that adenosine receptors that modulate membrane phosphoinositide hydrolysis do not interfere with the generation of cyclic adenosine monophosphate (cAMP) in cerebral cortex slices (9). However, experiments with GH_3 cell lines showed that R-PIA administration inhibited the release of prolactin induced by thyrotropin releasing hormone (TRH) by blocking the synthesis of phosphatidylinositol and cAMP (10).

The participation of adenosine in pituitary gonadotropin secretion has not been fully clarified. In previous studies we demonstrated that adenosine causes a dose-dependent reduction of basal LH and FSH or LHRH-stimulated secretion by hemipituitaries *in vitro* (11). The inhibitory effect of adenosine was potentiated by the simultaneous addition of dipyridamole, a blocker of adenosine reuptake by the cell, demonstrating that the purinergic action may have resulted from the activation of outer membrane receptors (12). Although we did not observe any effect of dipyridamole alone, it is probable that adenosine may be released by pituitary cells. Other investigators have detected adenosine release (100 nM) into the incubation bath, associated with the release of the enzyme adenosine deaminase in preparations of cells of the GH_4C_1 line, showing that the levels of released adenosine can be regulated within strict limits (13).

In the present study we report results suggesting the presence of subtype A_1 adenosine receptors in the gonadotrope and their involvement in the synthesis of membrane phosphoinositides.

Material and Methods

Male Wistar rats (200 to 220 g) housed in collective cages, at controlled temperature (22 to 24°C) and with 14 h of light and 10 h of dark, with free access to solid food and water, were used.

Drugs and solutions

The drugs used in this study were obtained from the following laboratories: Research Biochemicals Incorporated (RBI, Natick, MA, USA): 5-N-methylcarboxamidoadenosine (MECA) (A-024), R-isomer of phenylisopropyladenosine (A-009), 8-cyclopentyltheophylline (CPT) (C-102); Sigma Chemical Co. (St. Louis, MO, USA): phospholipase C (P4014); Merck (Frankfurter Strabe 250, Darmstadt, Germany): LiCl, myo-inositol. The nutrient solution (pH 7.4) consisted of Earle salt solution supplemented with 0.1% bovine serum albumin (BSA) and 15 mM (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) (HEPES).

Experimental procedures

After a period of adaptation to the laboratory of approximately 1 h, the animals were sacrificed by decapitation at 10:00 a.m. in all experiments. The brain was removed and the anterior pituitary dissected *in situ*. The anterior pituitary was bisected longitudinally and immersed into refrigerated nutrient solution (4°C). Each hemipituitary was placed in an incubation flask containing 1 ml of nutrient solution (37°C). After 1 h of pre-incubation in a Dubnoff metabolic shaker (80 cycles/min) for washing and stabilization of basal hormonal secretion levels, the medium was replaced with 1 ml of fresh solution containing the test substances. After 60 min of incubation the samples were collected in chilled plastic tubes and kept at -20°C for later determination of LH and FSH by radioimmunoassay. The hemipituitaries were weighed and hormone concentrations in the nutrient solution were expressed as ng/mg tissue weight. At the end of each experiment, 56 mM KCl was added to evaluate the functional viability of cells in the preparation on the basis of LH and FSH release from intracellular stores. The cells maintained their secretory response for more than 135 min of incubation, thus guaranteeing the viability of the preparation (data not shown).

Radioimmunoassay

LH and FSH concentrations in the nutrient solution were determined by double-antibody radioimmunoassay (RIA) (14). The hormones for radioiodination and specific antibodies were obtained from the National Institute of Arthritis, Diabetes and Digestive Diseases (NADDK, Baltimore, MD, USA) Rat Pituitary Hormone Program.

Statistical analysis

Data are reported as means ± SEM and were analyzed using the GBSTAT computer program. Statistical analysis was performed by analysis of variance (ANOVA), with the level of significance set at $P<0.05$ with the Newman-Keuls test.

Results

Inhibition of LH and FSH secretion induced by the activation of A_1 receptors by R-PIA or MECA

Increasing R-PIA concentrations (1 nM, 10 nM, 100 nM, 1 µM and 10 µM) caused a substantial and graded reduction of LH and FSH release. This effect was dose dependent and the maximum inhibition was reached with the 1 µM dose. The dose-response curves for the two hormones were similar in terms of the pattern of inhibition observed (Figure 1A,B).

Previous incubation for 30 min with increasing doses of CPT (10 nM, 100 nM, 1 µM and 10 µM), an A_1 receptor-specific antagonist, was performed to determine which dose would block the response induced by the addition of R-PIA to the nutrient solution. Treatment with the antagonist alone had no effect on basal LH or FSH

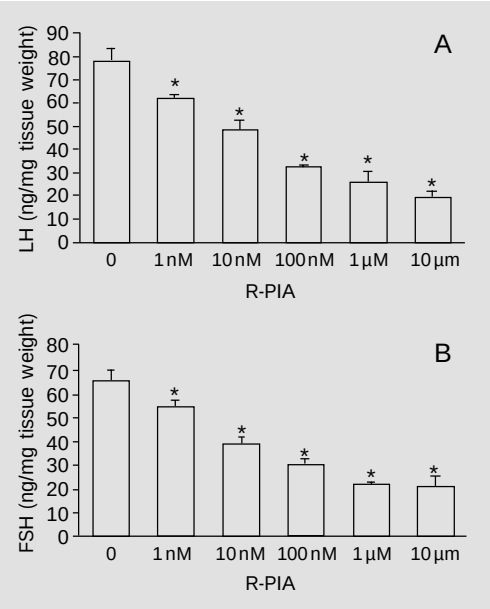

Figure 1 - Effects of A_1 receptor activation by different concentrations of R-PIA on basal LH (A) and FSH (B) secretion. Wistar rat hemipituitaries were pre-incubated for 60 min for stabilization of preparation and incubated in new Earle salt solution supplemented with 0.1% BSA and 15 mM HEPES at 37°C, pH 7.4, in a Dubnoff metabolic shaker (80 cycles/min) for a further 60 min. Data are reported as means ± SEM (N = 5). *P<0.05 compared to control (0) (Newman-Keuls test).

levels at any of the doses tested. Under conditions of equimolality, the antagonist did not block the inhibitory effects of R-PIA on hormonal secretion. Partial blockade occurred after administration of 100 nM CPT at 10 times higher concentration. Only at a 100 times higher concentration (1 µM) did we observe total blockade of the agonist effects on LH and FSH secretion (Figure 2A,B).

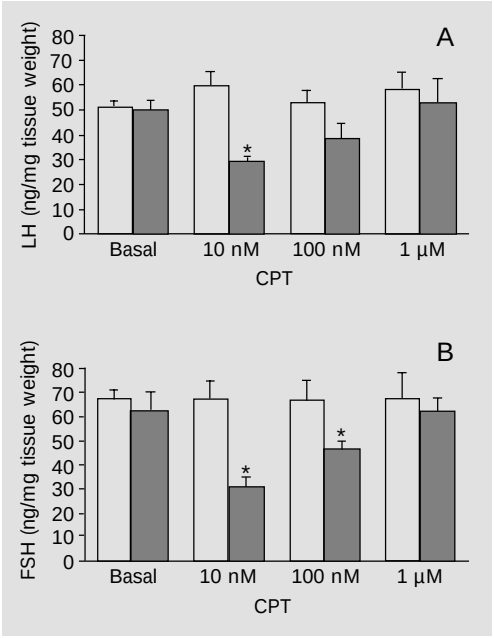

Figure 2 - Effects of previous incubation (30 min) with CPT, an A_1 antagonist, on LH (A) and FSH (B) secretion inhibited by 10 nM R-PIA (closed bars). The preparation conditions are the same as described in the legend to Figure 1. Data are reported as means ± SEM (N = 5). *P<0.05 compared to control (basal) (Newman-Keuls test).

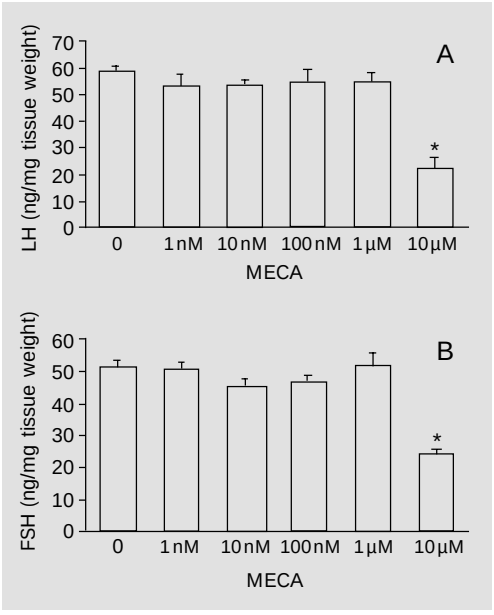

Figure 3 - Effects of administration of different MECA concentrations on basal LH (A) and FSH (B) secretion. The preparation conditions are the same as described in the legend to Figure 1. Data are reported as means ± SEM (N = 5). *P<0.05 compared to control (0) (Newman-Keuls test).

Administration of MECA at concentrations of 1 µM or less did not induce any changes in basal LH and FSH secretion. A significant inhibition of approximately 50% of LH and FSH secretion occurred when MECA was added at the dose of 10 µM (Figure 3A,B).

Effects of R-PIA administration on LH and FSH secretion stimulated by phospholipase C

In this experiment we used phospholipase C to determine whether the inhibitory effect of R-PIA on LH and FSH secretion continued after the activation of inositol triphosphate and diacylglycerol promoted by this enzyme. The addition of phospholipase C (0.5 IU/ml) to the incubation medium induced a substantial increase in basal LH and FSH levels in the nutrient solution. When phospholipase C was added in combination with different doses of R-PIA (1 nM, 10 nM, 100 nM, 1 µM and 10 µM) there was a dose-dependent decrease in LH and FSH secretion stimulated by phospholipase C (Figure 4A,B).

Effects of adenosine administration on LH and FSH secretion inhibited by LiCl

To determine whether functional impairment of the cycle of membrane phosphoinositide synthesis interferes with basal or adenosine-inhibited LH and FSH secretion, we added 5 mM LiCl alone or in combination with 10 nM adenosine to the incubation medium. LiCl induced a significant decrease in basal LH and FSH secretion but had no effect on the secretion of these hormones when added in combination with 1 mM myo-inositol. This substrate, in turn, had no effect on LH and FSH secretion when added alone to the preparation. The administration of 10 nM adenosine alone or in combination with 5 mM LiCl elicited a significant decrease in basal LH and FSH secretion, with no significant differences between these effects (Figure 5A,B).

Discussion

The effects of R-PIA were similar for LH and FSH secretion, suggesting the existence of a single purinergic regulatory mechanism for both hormones (Figure 1A,B). This hypothesis is supported by the similar behavior resulting from the blockade of these effects induced by previous administration of CPT, a specific A_1 receptor antagonist (Figure 2A,B). This blockade demonstrates the existence of A_1 receptors in the gonadotropes. The lack of effect of CPT on basal LH and FSH secretion suggests the absence of significant actions of endogenous adenosine in this type of experiment. On the other hand, studies with cultures of GH_4C_1 cell lines demonstrated adenosine release accompanied by adenosine deaminase release under basal experimental conditions. Besides, an increase in prolactin secretion was found when the enzyme was added alone, suggesting that released adenosine may have a tonic-inhibitory autocrine action (13). The presence of an inhibitory effect of the A_2 receptor agonist MECA on LH and FSH secretion only with the use of supramicromolar concentrations may be by an effect on A_1 receptors. The lack of effect of MECA at lower concentrations, where it would selectively activate A_2 receptors, suggests the probable absence of A_2 receptors in the gonadotrope (Figure 3A,B).

The increased LH and FSH basal secretion observed after the addition of phospholipase C (Figure 4A,B) or the inhibition induced by the addition of LiCl (Figure 5A,B) to the medium supports other findings indicating the contribution of membrane phosphoinositides as cell messengers to the regulation of the basal secretion of these hormones (15). The progressive and dose-related inhibition of phospholipase C-stimulated LH and FSH secretion by R-PIA suggests that the activation of A_1 receptors may inhibit the increase in LH and FSH secretion by a mechanism not dependent on the secretory effects of membrane phosphoinositides.

The inhibition of LH and FSH secretion induced by the administration of adenosine mimicked the effect of R-PIA, suggesting that the nucleoside may regulate gonadotropin secretion by a mechanism depending on A_1 receptor activation in the gonadotrope. The fact that there were no significant differences between the effects obtained by the

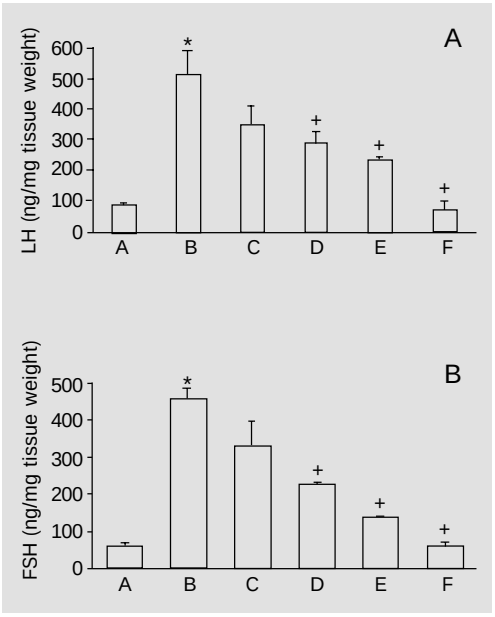

Figure 4 - Effects of administration of different R-PIA doses on LH (A) and FSH (B) secretion stimulated by phospholipase C (0.5 IU/ml). The preparation conditions are the same as described in the legend to Figure 1. Data are reported as means ± SEM (N = 5). *P<0.01, +P<0.05 compared to control (A) (Newman-Keuls test).

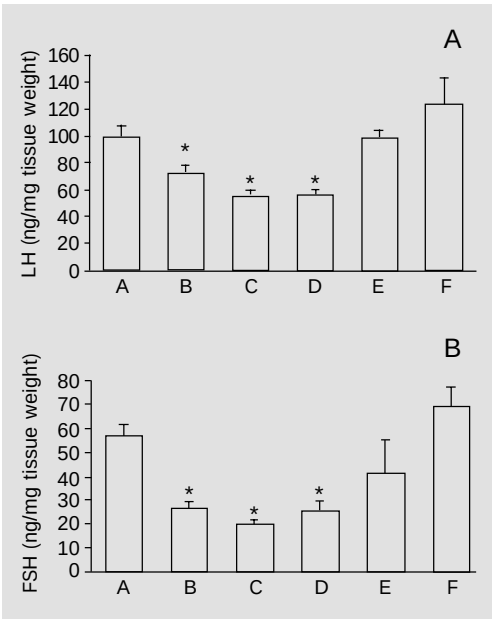

Figure 5 - Effects of administration of 0.01 µM adenosine on LH (A) and FSH (B) secretion inhibited by 5 mM LiCl. The preparation conditions are the same as described in the legend to Figure 1. A = Basal, B = 10 nM adenosine, C = 5 mM LiCl + 10 nM adenosine, D = 5 mM LiCl, E = 5 mM LiCl + 1 mM myo-inositol, F = 1 mM myo-inositol. Data are reported as means ± SEM (N = 5). *P<0.05 compared to control (A) (Newman-Keuls test).

combination of adenosine + LiCl and those obtained by the separate addition of each substance impairs the interpretation of the results.

Among the possible mechanisms that may mediate the inhibition of gonadotropin induced by the activation of A_1 receptors, the one based on membrane G_iP (guanine nucleotide-binding membrane proteins) inhibition may best fit the present case. G_iP inhibition may result in a decrease of both adenyl cyclase and phospholipase C activity.

Recent studies have demonstrated that adenosine inhibits prolactin secretion by acting on a purinergic receptor that modulates the activity of the enzymes adenyl cyclase and phospholipase C by a mechanism depending on membrane G_iP activation (16). Other studies have shown that administration of R-PIA inhibits the release of PRL induced by TRH by inhibiting the decrease in phosphatidylinositol and cAMP synthesis in GH_3 cell lines (10).

An alternative hypothesis is the blockade of Ca^{2+} influx or the increased Ca^{2+} efflux from the cell directly mediated at the membrane GP level. It has been demonstrated that the activation of A_1 receptors reduced the concentration of basal cytoplasmic Ca^{2+} and of Ca^{2+} stimulated by protein kinase C in GH_3B_6 cell lines (17), suggesting that the inhibition may depend on the firing of a mechanism preceding the activation of this kinase. Ca^{2+} efflux from the cell may also be mediated by GP after A_1 receptor activation, a mechanism based on Ca^{2+} exchange with Na^+ and depending on a pathway sensitive to pertussis toxin (4). On the other hand, it has been recently demonstrated that A_1 receptors decrease intracellular free calcium. This would induce a decrease in nitric oxide (NO) synthase activation in the gonadotropes, resulting in decreased NO synthesis. NO stimulates LH and FSH release by activating guanylate cyclase that synthesizes cGMP from GTP. cGMP concentrations increase in the gonadotropes inducing activation of LH and FSH release. Adenosine decreases NO release and concentration in the gonadotrope cells, as well as cGMP formation followed by a consequent decrease of LH and FSH release (18).

In conclusion, the present results show that the activation of A_1 receptors by R-PIA induced a dose-dependent inhibition of pituitary LH and FSH secretion by a mechanism not depending on increased membrane phosphoinositide synthesis induced by phospholipase C, leading us to formulate the hypotheses that this inhibition may have occurred by mediation of a membrane G_iP which, once activated by A_1 receptors, may act at different levels, inhibiting cAMP and phosphoinositide synthesis, and Ca^{2+} influx, or stimulating Ca^{2+} efflux from the cell. Aside from these preliminary speculations, the mechanisms involved in signal transduction by the activation of A_1 receptors remain obscure and further experiments are needed to clarify the mechanisms.

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