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Adenosine A_1 receptor-mediated inhibition of *in vitro* prolactin secretion from the rat anterior pituitary

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Abstract

In previous studies, we demonstrated biphasic purinergic effects on prolactin (PRL) secretion stimulated by an adenosine A_2 agonist. In the present study, we investigated the role of the activation of adenosine A_1 receptors by (R)-N^6-(2-phenylisopropyl)adenosine (R-PIA) at the pituitary level in *in vitro* PRL secretion. Hemipituitaries (one per cuvette in five replicates) from adult male rats were incubated. Administration of R-PIA (0.001, 0.01, 0.1, 1, and 10 μM) induced a reduction of PRL secretion into the medium in a U-shaped dose-response curve. The maximal reduction was obtained with 0.1 μM R-PIA (mean ± SEM, 36.01 ± 5.53 ng/mg tissue weight (t.w.)) treatment compared to control (264.56 ± 15.46 ng/mg t.w.). R-PIA inhibition (0.01 μM = 141.97 ± 15.79 *vs* control = 244.77 ± 13.79 ng/mg t.w.) of PRL release was blocked by 1 μM cyclopentyltheophylline, a specific A_1 receptor antagonist (1 μM = 212.360 ± 26.560 ng/mg t.w.), whereas cyclopentyltheophylline alone (0.01, 0.1, 1 μM) had no effect. R-PIA (0.001, 0.01, 0.1, 1 μM) produced inhibition of PRL secretion stimulated by both phospholipase C (0.5 IU/mL; 977.44 ± 76.17 ng/mg t.w.) and dibutyryl cAMP (1 mM; 415.93 ± 37.66 ng/mg t.w.) with nadir established at the dose of 0.1 μM (225.55 ± 71.42 and 201.9 ± 19.08 ng/mg t.w., respectively). Similarly, R-PIA (0.01 μM) decreased (242.00 ± 24.00 ng/mg t.w.) the PRL secretion stimulated by cholera toxin (0.5 mg/mL; 1050.00 ± 70.00 ng/mg t.w.). In contrast, R-PIA had no effect (468.00 ± 34.00 ng/mg t.w.) on PRL secretion stimulation by pertussis toxin (0.5 mg/mL; 430.00 ± 26.00 ng/mg t.w.). These results suggest that inhibition of PRL secretion after A_1 receptor activation by R-PIA is mediated by a G_i protein-dependent mechanism.

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Key words
- Cyclopentyltheophylline
- Dibutyryl cAMP
- R-PIA
- Pertussis toxin
- Cholera toxin

Introduction

The modulation of the activity of adenyl cyclase is one of the main functions described for purinergic mechanisms involving type A_1 and A_2 outer membrane receptors (1). The ability of A_1 and A_2 receptors to inhibit and activate the enzyme, respectively, has also been used for some time to identify and differentiate adenosine receptors (2). An alternative approach to the classification of adenosine receptors is based on the premise that A_1 receptors produce more potent responses to purine-derived analogues with substitutions at the N^6 position, such as (R)-N^6-(2-phenylisopropyl) adenosine (R-PIA), than to carboxamide analogues such as 5'-(N-ethylcarboxyamido) adenosine (3).

The involvement of guanine nucleotide-binding proteins (GNBP) in the signal transduction following activation of A_1 receptors has expanded the list of probable receptor subtypes. The classification of these subtypes has been based on their blockade by pertussis toxin (PTX) in neurons (4). From this viewpoint, activation of A_{1A}, A_{1C} and A_{1B} receptors may regulate Ca^{2+} influx, K^+ efflux and the inhibition of adenyl cyclase, respectively. Moreover, all of these mechanisms may depend on the activation of membrane GNBP, since coupling between the A_1 receptor and G_0 and G_i proteins has been characterized using R-PIA as the binding agonist (5). Outflow of Ca^{2+} from the cell may also be mediated by GNBP following the activation of A_1 receptors, a mechanism that may use Ca^{2+}/Na^+ exchange and depend on a PTX-sensitive pathway (6). On the other hand, purinergic activation mechanisms have been related to inositol triphosphate synthesis because studies of isolated sympathetic ganglion have demonstrated that endogenously released adenosine can inhibit postsynaptic stimulation and turnover of myo-inositol (7).

Activation of A_1 receptors can also induce responses mediated by mechanisms based on the activation of different membrane GNBP. Some studies have shown that R-PIA inhibits the release of prolactin (PRL) induced by thyrotropin releasing hormone by blocking the synthesis of phosphatidylinositol and cAMP in GH_3 cell lines (8). A probable mechanism of autocrine regulation of PRL secretion by adenosine has been suggested on the basis of results obtained with GH_4C_1 cell lines (9). Both adenosine and adenosine deaminase were released into the medium, indicating that the levels of adenosine released may be controlled within narrow limits.

In the present study, we analyzed possible mechanisms of signal transduction involving the participation of G_s or G_i proteins, and the synthesis of membrane phosphoinositide and cAMP triggered by A_1 receptor activation in preparations of isolated hemipituitaries.

Material and Methods

We used male Wistar rats weighing 200 to 220 g from the central Animal House of the Ribeirão Preto, Faculty of Medicine (University of São Paulo). The animals were kept in collective cages in an artificially controlled environment with a temperature of 22-24ºC and 14 h of light (7:00-21:00). Animals had free access to food and tap water.

Drugs and solutions

Phospholipase C, dibutyryl cyclic AMP (dcAMP), PTX, cholera toxin (CTX), bovine serum albumin, and HEPES were obtained from Sigma (St. Louis, MO, USA). The other substances - R-PIA and cyclopentyltheophylline (CPT) - were provided by Research Biochemicals Incorporated (Natick, MA, USA). The nutrient solution consisted of Earle's salt solution containing 0.1% bovine serum albumin and 15 mM HEPES at pH 7.4.

Experimental procedures

In all experiments, the animals were brought into the laboratory for a period of adaptation of approximately 1 h in order to eliminate possible interference due to stress prior to decapitation at 10:00 am. The brain was then removed, the neural lobe discarded and the anterior pituitary dissected *in situ*, divided longitudinally into two approximately equal parts, and immersed in nutrient solution (4ºC). Each hemipituitary was transferred in a random sequence to an individual cuvette (assayed with five replicates per group) containing 1 mL nutrient solution (37ºC) and then incubated for 60 min in a Dubnoff metabolic shaker with constant shaking (50 cycles per min, 95% O_2/5% CO_2) for washing and for the stabilization of basal hormone secretion levels. All tests were conducted immediately after the preincubation period except for the PTX and CTX experiments. All concentrations of the tested drugs used were based on their effects in similar experimental situations. In the experiment in which PTX and CTX were added, the preliminary incubation lasted 180 min, during which the nutrient solution was replaced at 60-min intervals. After incubation, samples were placed in plastic tubes at -20ºC and the hemipituitaries were weighed on a torsion scale. The recorded concentration of PRL released into the nutrient solution was divided by the weight (mg) of the respective hemipituitary and is reported 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 the release of PRL from intracellular stores. A significant increase in basal PRL secretion was recorded (basal = 270 ± 30 *vs* KCl = 1350 ± 52 ng/mL, $P < 0.001$) demonstrating that the cells maintained their secretory response for more than 195 min of incubation, thus guaranteeing the viability of the preparation.

Radioimmunoassay

PRL concentrations in the nutrient solution were determined by double-antibody radioimmunoassay (10). The hormones for radioiodination and specific antibodies were obtained from the National Institute of Arthritis, Diabetes and Digestive Diseases (NIDDK, Baltimore, MD, USA) Rat Pituitary Hormone Program. All the samples from one experimental group (control versus respective groups) were analyzed in a single assay. The intra- and inter-assay variations for the PRL assay were, 2.57 and 17.08%, respectively.

Statistical analysis

Data are reported as means ± SEM. The significance of differences in sequential changes in hormone levels between groups was determined by one-way ANOVA followed by the Bonferroni (for comparisons between groups with one treatment only) or Newman-Keuls (for groups with two or more treatments) *post hoc* test. The level of significance was set at $P < 0.05$.

Results

Effects of R-PIA on basal PRL secretion

As shown in Figure 1, R-PIA induced a significant reduction in PRL release at all concentrations used (0.001, 0.01, 0.1, 1, and 10 μM) in comparison with those of the basal group. The dose-effect relationship was linear up to 0.1 μM, which induced a decrease of approximately 80% in basal PRL levels. At subsequent doses (1 and 10 μM), there was a reduction in inhibitory power, pointing to a biphasic pattern in the secretory response.

Increasing doses of CPT (0.01, 0.1, and 1 μM), a specific A_1 receptor antagonist, were administered 30 min before incubation with R-PIA to determine which dose would block

the response to the latter. Under equimolar conditions (0.01 μM), the antagonist caused only a partial blockade of the inhibitory effects of R-PIA on PRL secretion. Total blockade of the effects of the agonist on PRL secretion was only observed at a dose 100 times higher - 1 μM. Administration of the antagonist alone had no effect on basal PRL levels at any dose used (Figure 2).

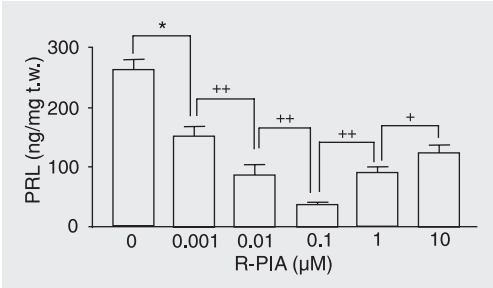

Figure 1. Effect of the activation of A_1 receptors by different concentrations of (R)-N^6-(2-phenylisopropyl)adenosine (R-PIA) on basal prolactin (PRL) secretion. Data are reported as means ± SEM ng PRL/mg tissue weight (t.w.) for 5 replicates. *P < 0.01 compared to the untreated (0) group; $^{+}$P < 0.05 and $^{++}$P < 0.001 for the comparisons indicated in the figure (ANOVA followed by the Bonferroni multiple comparison test).

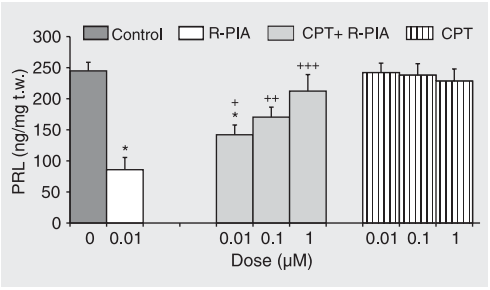

Figure 2. Effect of prior (30 min) administration of cyclopentyltheophylline (CPT) associated or not with 0.01 μM (R)-N^6-(2-phenylisopropyl)adenosine (R-PIA) on prolactin (PRL) secretion. Data are reported as means ± SEM ng PRL/mg tissue weight (t.w.) for 5 replicates. *P < 0.05 compared to the untreated group; $^{+}$P < 0.05, $^{++}$P < 0.01 and $^{+++}$P < 0.001 compared to CPT-treated groups (ANOVA followed by the Newman-Keuls multiple comparison test).

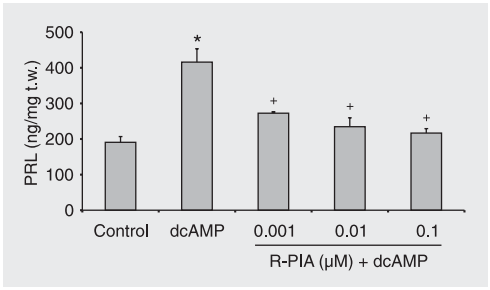

Figure 3. Effect of the administration of (R)-N^6-(2-phenylisopropyl)adenosine (R-PIA) associated or not with 1000 μM dibutyryl cAMP (dcAMP) on prolactin (PRL) secretion. Data are reported as means ± SEM ng PRL/mg tissue weight (t.w.) for 5 replicates. *P < 0.001 compared to untreated group. $^{+}$P < 0.001 (dcAMP *vs* dcAMP + R-PIA group). Newman-Keuls multiple comparison test was used after ANOVA.

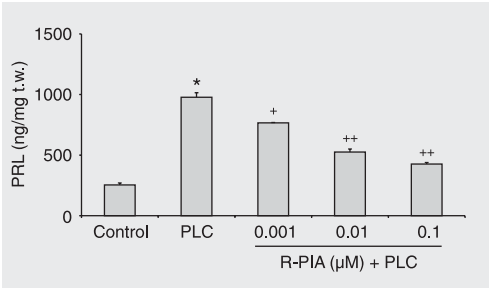

Figure 4. Effect of the administration of (R)-N^6-(2-phenylisopropyl)adenosine (R-PIA) associated or not with 0.5 IU/mL phospholipase C (PLC) on prolactin (PRL) secretion. Data are reported as means ± SEM ng PRL/mg tissue weight (t.w.) for 5 replicates. *P < 0.001 compared to untreated group. $^{+}$P < 0.01 and $^{++}$P < 0.001 (PLC *vs* PLC + R-PIA group) (ANOVA followed by the Newman-Keuls multiple comparison test).

Effects of R-PIA on PRL secretion stimulated by dibutyryl cAMP

A cAMP analogue, dcAMP, that mimics the intracellular effects of cAMP, was used in an attempt to confirm that the inhibition of PRL secretion induced by the activation of A_1 receptors was independent of the increase in cellular cAMP levels. Administration of dcAMP (1 mM) produced a sharp increase (1.5 times) in basal PRL levels. The administration of increasing doses of R-PIA up to 0.1 μM inhibited the PRL secretion induced by dcAMP. At the highest dose (1 μM), the expected increase in the response to the concomitant addition of dcAMP was blocked completely (Figure 3).

Effects of R-PIA on PRL secretion stimulated by phospholipase C

We used phospholipase C to determine whether the inhibitory effect of R-PIA on PRL secretion was altered by activation of the synthesis 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 PRL levels. A dose-dependent decrease in phospholipase C-stimulated PRL secretion was observed after combination of the compound with increasing doses of R-PIA (Figure 4). At the highest dose (1 μM), R-PIA prevented any increase in PRL levels expected in response to the action of the enzyme.

Effects of R-PIA administration on PRL secretion stimulated by pertussis toxin and cholera toxin

The irreversible blockade of the cycle of

G_i and G_s protein activation induced by PTX and CTX, respectively, was tested to determine whether the inhibitory effect of R-PIA on PRL secretion in response to A_1 activation was dependent on the integrity of this pathway. A significant increase in the medium concentration of PRL was observed 180 min after administration of 0.5 mg/mL CTX or PTX (Figure 5). R-PIA (0.01 µM) inhibited the secretion of PRL stimulated by CTX, but had no effect on the secretory response of this hormone to PTX administration (Figure 5).

Discussion

We demonstrated the *in vitro* inhibition of PRL secretion by the anterior pituitary gland in response to graded doses of R-PIA. The doses of 1 and 10 µM caused a decrease that was lower than the maximum inhibition produced by the 0.1-µM dose (Figure 2), indicating a biphasic pattern of response (U-shaped dose-response curve). These results compare well with our earlier report of biphasic purinergic effects on PRL secretion stimulated by the adenosine agonist ($A_2 > A_1$) 5-(N-methylcarboxyamido)adenosine (MECA) (11). We showed that the 10 µM dose had only a residual stimulatory effect on PRL secretion in hemipituitaries incubated *in vitro* compared with 1 µM (maximum stimulatory response). This suggests the co-existence of A_2 and A_1 adenosine receptors in the PRL-secreting cells.

This biphasic pattern was confirmed in the present study by the addition of R-PIA, an $A_1 > A_2$ agonist, given that the lower concentrations brought about a dose-related reduction in PRL secretion, whereas higher concentrations were relatively less potent. Other investigators have shown that R-PIA influences basal adenyl cyclase activity, exhibiting inhibitory effects at low concentrations, but stimulatory effects at higher ones. A similar tendency was found in PRL secretion, since low concentrations of R-PIA reduced both basal and thyrotropin releasing hormone-stimulated PRL release, whereas higher concentrations restored PRL release in perifused pituitaries (12).

The use of CPT, a specific A_1 receptor antagonist, proved to be effective in blocking the R-PIA-induced inhibition of PRL secretion only at a 100-times higher MECA dose. This antagonist alone did not produce any variation in basal PRL secretion at any of the concentrations used. These data indicate that low R-PIA concentrations have specific effects that may be mediated by the activation of A_1 adenosine receptors. In addition, the fact that CPT caused no change in basal PRL levels suggests that the adenosine released at this time did not produce perceptible autocrine regulatory effects in this type of preparation (Figure 2).

In a similar study, we showed that caffeine, another xanthinic antagonist, had no effect on basal PRL secretion in the hemipituitary *in vitro*, but efficiently blocked adenosine-induced secretion (13). This suggests that adenosine has a regulatory action on PRL release via the A_2 receptor, but not under basal conditions. This evidence is reinforced by the fact that two selective A_1 adenosine receptor antagonists and a highly specific A_2 receptor antagonist had no effect on basal PRL release (14).

Indeed, studies of GH_4C_1 cell line cultures have shown the release of adenosine accompanied by the enzyme adenosine deaminase under basal experimental conditions (9). The addition of this enzyme alone

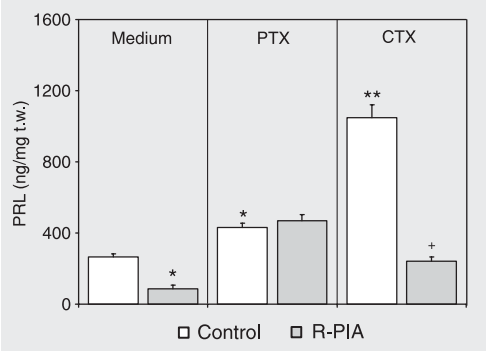

Figure 5. Effect of the administration of 0.01 µM (R)-N^6-(2-phenylisopropyl)adenosine (R-PIA) on the prolactin (PRL) secretion induced by 0.3 µg/mL pertussis toxin (PTX) or 6.0 µg/mL cholera toxin (CTX) (pre-incubated for 180 min). Data are reported as means ± SEM ng PRL/mg tissue weight (t.w.) for 5 replicates. *P < 0.01 and **P < 0.001 compared to the untreated group; +P < 0.001 compared to the treated group (ANOVA followed by the Newman-Keuls multiple comparison test).

increased PRL secretion, suggesting that the adenosine released may have a tonic-inhibitory autocrine action (9,15). These findings support the hypothesis that the activation of A_1 adenosine receptors may result in a reduction of PRL secretion, and that this effect may depend on the levels of adenosine deaminase in the medium.

The increase in PRL secretion observed after the addition of both dcAMP (Figure 3) and phospholipase C (Figure 4) supports the evidence from other studies which have demonstrated the contribution of cAMP and membrane phosphoinositides to the regulation of basal or induced PRL secretion (16,17). The R-PIA inhibition of both the dcAMP- and phospholipase C-induced PRL secretion suggests that the A_1 receptors may inhibit PRL secretion through a mechanism independent of the corresponding second messengers (i.e., cAMP and Ca^{2+} mobilization).

Our data suggest that the inhibition induced by activation of A_1 receptors depends on the activation of G_i protein, but not G_s protein, given that R-PIA inhibited PRL secretion stimulated by CTX, but had no effect under stimulation by PTX (Figure 5). Several studies have shown a similar role of PTX in preventing the dopamine-induced inhibition of PRL secretion in adenohypophyseal cell cultures (18). An alternative interpretation would be the blockade of Ca^{2+} influx or an increase in Ca^{2+} efflux from the cell mediated at the level of membrane GNBP. Some studies have shown that activation of A_1 receptors decreased basal or protein kinase C-stimulated intracellular Ca^{2+} release in a GH_3B_6 cell line (19), suggesting that this inhibition may depend on the triggering of a mechanism that precedes activation of this kinase. Ca^{2+} outflow from the cell may also be mediated by GNBP after A_1 receptor activation, a mechanism that would involve exchange with Na^+ and be dependent on a pathway sensitive to PTX (6).

Our results show that the activation of A_1 receptors by R-PIA inhibits pituitary PRL secretion in a dose-dependent manner through a mechanism that is dependent on the blockade of G_i protein. By contrast, this mechanism is not dependent on the blockade of the secretory effect via GNBP caused by either cAMP or membrane phosphoinositides.

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