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Physiology

Adenosine acts by A_1 receptors to stimulate release of prolactin from anterior-pituitaries *in vitro*

(follicle-stimulating hormone/luteinizing hormone/nitric oxide synthase/cGMP/folliculostellate cells)

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ABSTRACT **Adenosine has been identified in the anterior pituitary gland and is secreted from cultured folliculostellate (FS) cells. To determine whether adenosine controls the secretion of anterior pituitary hormones *in vitro*, adenosine was incubated with anterior pituitaries. It stimulated prolactin (PRL) release at the lowest concentration used (10^{-10} M); the stimulation peaked at 10^{-8} M with a threefold increase in release and declined to minimal stimulation at 10^{-4} and 10^{-3} M. Follicle-stimulating hormone release was maximally inhibited at 10^{-8} M, whereas luteinizing hormone release was not significantly inhibited. Two selective A_1 adenosine receptor antagonists (10^{-7} or 10^{-5} M) had no effect on basal PRL release, but either antagonist completely blocked the response to the most effective concentration of adenosine (10^{-8} M). In contrast, a highly specific A_2 receptor antagonist (10^{-7} or 10^{-5} M) had no effect on basal PRL release or the stimulation of PRL release induced by adenosine (10^{-8} M). We conclude that adenosine acts to stimulate PRL release *in vitro* by activating A_1 receptors. Since the A_1 receptors decrease intracellular-free calcium, this would decrease the activation of nitric oxide synthase in the FS cells, resulting in decreased release of nitric oxide (NO). NO inhibits PRL release by activating guanylate cyclase that synthesizes cGMP from GTP; cGMP concentrations increase in the lactotrophs leading to inhibition of PRL release. In the case of adenosine, NO release from the FS cells decreases, resulting in decreased concentrations of NO in the lactotrophs, consequent decreased cGMP formation, and resultant increased PRL release.**

In 1989 Gonzalez *et al.* (1) presented evidence for a substance of pituitary origin that acts as a trophic agent for hypothalamic dopaminergic neurons. Their findings included the observation that implantation of anterior pituitaries into the brain of rats increased secretory activity of hypothalamic dopaminergic neurons. The secretion of dopamine by dispersed hypothalamic cells in culture was later found to be increased by coincubation with pituitary cells (J. C. Porter, unpublished observations) or by inclusion of pituitary extract in the medium (2). This substance was named pituitary cytotrophic factor (3). Cytotrophic factor produced a dose- and time-related increase in dopamine release as well as the quantity of tryosine hydroxylase mRNA and the enzyme itself. Cytotrophic factor was subsequently identified as adenosine by Porter *et al.* (4). They hypothesized that adenosine secreted by pituitary cells reached the hypothalamus by retrograde blood flow in the pituitary stalk vasculature (5), where it increased the secretory activity of dopaminergic neurons.

Adenosine was secreted by folliculostellate (FS) cells cloned from a pituitary tumor (J. C. Porter, unpublished observations). Therefore, it is plausible that adenosine secreted by such cells may affect the secretion of other pituitary cells. To test this possibility, we conducted studies on the effect of adenosine on the secretion of prolactin (PRL), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) by anterior pituitary tissue *in vitro*.

Four adenosine receptor subtypes, termed A_1, A_{2a}, A_{2b}, and A_3, have been characterized (6), and each subtype is present in neural tissue. A_1 and A_{2a} receptors have high affinity, whereas A_{2b} and A_3 receptors have low affinity, for adenosine. Consequently, we attempted to determine the receptor subtype responsible for the remarkable PRL-releasing action of adenosine by evaluating the effects of adenosine in the presence or absence of potent, selective A_1 and A_{2a} receptor antagonists.

METHODS

Adult male rats of the Sprague Dawley strain (Holtzmann, Madison, WI) (200–250 g) were housed two per cage under controlled conditions of temperature (23–25°C) and lighting (on 5:00–17:00 h). The animals had free access to a pellet diet and tap water.

Incubation of Anterior Pituitaries. The method is similar to that recently reported (7). In brief, after removal of the posterior lobe, the anterior pituitary was bisected longitudinally and each half was incubated in a tube containing 1.0 ml of Krebs–Ringer bicarbonate (1 mg/ml ascorbic acid; pH 7.4) buffer [Krebs–Ringer buffer (KRB)] in an atmosphere of 95% O_2/5% CO_2 in a Dubnoff shaker (50 cycles per min) for a period of 60 min. After this preincubation period, anterior pituitaries were incubated for 3 h in fresh KRB buffer alone or KRB containing graded concentrations of adenosine or adenosine receptor blockers. All experiments were replicated twice. The medium was then aspirated and stored frozen at −20°C until measurement of FSH, LH, and PRL by RIA using kits supplied by the National Institute of Arthritis, Digestive, Diabetes and Kidney Disease.

Drugs. Adenosine was purchased from Sigma. Adenosine receptor antagonists, 8-phenyltheophylline and 8-cyclopentyltheophylline, both A_1 antagonists, and 8-(3-chlorostyryl) caffeine, an A_2 antagonist, were purchased from Research Biochemicals (Natick, MA).

Statistics. Results were analyzed by one-way ANOVA with repeated measures, and significance of differences between group means was determined with the Student–Newman–

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Abbreviations: PRL, prolactin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; KRB, Krebs–Ringer buffer; FS, folliculostellate; NO, nitric oxide; NOS, nitric oxide synthase.

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Keuls test. The significance of differences between the means of two groups was calculated using Student's *t* test.

RESULTS

Effect of Adenosine on PRL Release. Adenosine produced a dose-related stimulation of PRL release with more than a twofold increase at the lowest concentration studied (10^{-10} M), which peaked at 10^{-8} M and then declined to minimal, but still significant, stimulatory effects at the highest two doses ($10^{-4}-10^{-3}$ M) (Fig. 1). Thus, there was a bell-shaped dose-response curve of adenosine stimulation of PRL release.

Effect of Adenosine on Gonadotropin Secretion. Adenosine had little effect on gonadotropin secretion. However, in the case of FSH, there was an apparent U-shaped dose-response curve of inhibition with the maximal effect at the same concentration that gave maximal stimulation of PRL release (10^{-8} M) (Fig. 2). In contrast, there was no significant effect of adenosine on LH release (Fig. 3).

Effect of Selective A_1 and A_{2a} Adenosine Receptor Antagonists on PRL Release and the Response to Adenosine. The highly selective A_1 adenosine receptor antagonist, 8-phenyltheophylline, in concentrations of $10^{-7}-10^{-5}$ M had no effect on PRL release, but it completely blocked PRL release induced by two concentrations of adenosine (10^{-8} and 10^{-5}M) (Fig. 4).

Another highly selective A_1 adenosine receptor antagonist, 8-cyclopentyltheophylline ($10^{-7}-10^{-5}$ M), again had no effect on basal PRL release, but it also completely blocked the stimulatory action of adenosine (10^{-8} M) (Fig. 5).

To determine the possible role of the A_2, adenosine receptors, a highly selective A_{2a} adenosine receptor antagonist, 8-(3-chlorostyryl) caffeine, had no effect on basal PRL release at concentrations of $10^{-7}-10^{-5}$ M. However, in contrast to the effects of A_1 receptor antagonists, it had no effect on the response to the most effective concentration of adenosine (10^{-8} M) (Fig. 6).

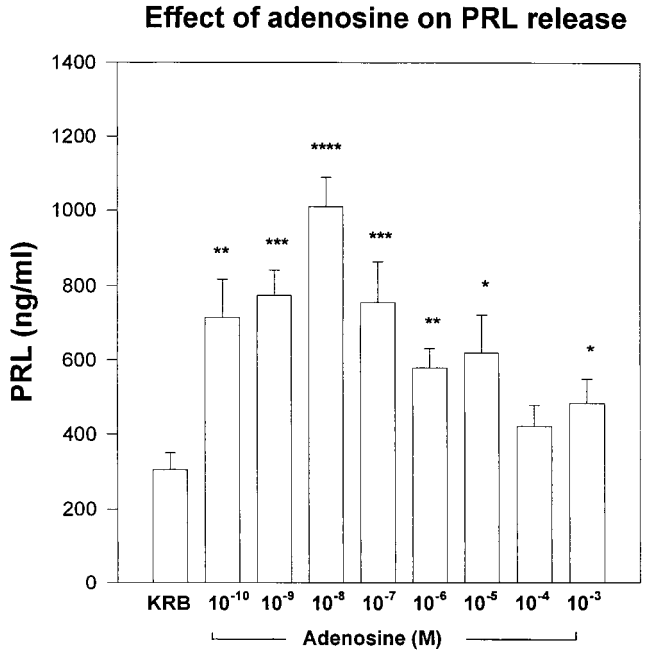

FIG. 1. The effect of adenosine on PRL release. In this and subsequent figures, the height of the column represents the mean ± 1 SEM. There were eight tubes containing one hemipituitary in each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ versus KRB controls in this and subsequent figures.

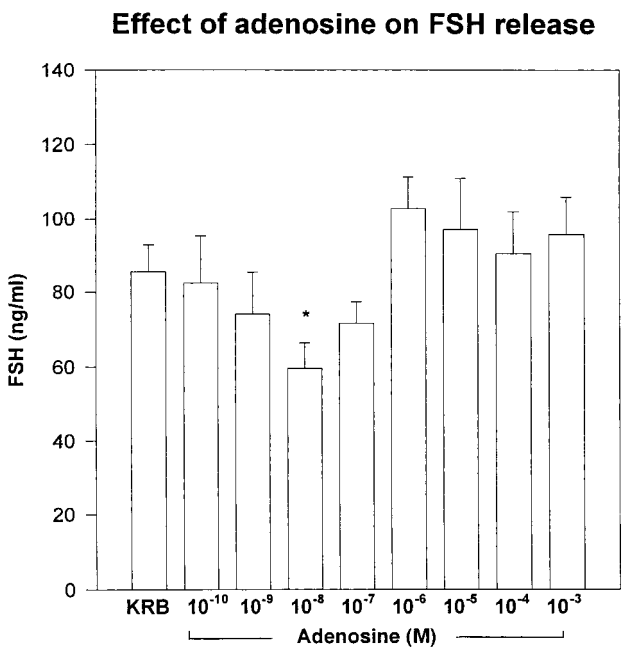

FIG. 2. The effect of adenosine on FSH release.

DISCUSSION

Adenosine appears to be the most potent stimulator of PRL release yet discovered. The relationship between adenosine concentrations and PRL release was bell shaped. The downslope in PRL release, which occurred with increasing adenosine concentrations during a 3-hr incubation period, may result from short-loop negative feedback of the released PRL (8). In spite of this feedback, PRL secretion did not return to basal values. Not only is adenosine a most potent stimulator of PRL release, but also it caused the largest increase in PRL we have seen *in vitro*, increasing PRL release by up to threefold. This occurred in spite of the removal of the normal hypothalamic inhibition of PRL release under *in vitro* conditions. In contrast, there was no significant effect of adenosine on LH release in the same experiments, but there was a U-shaped dose-response

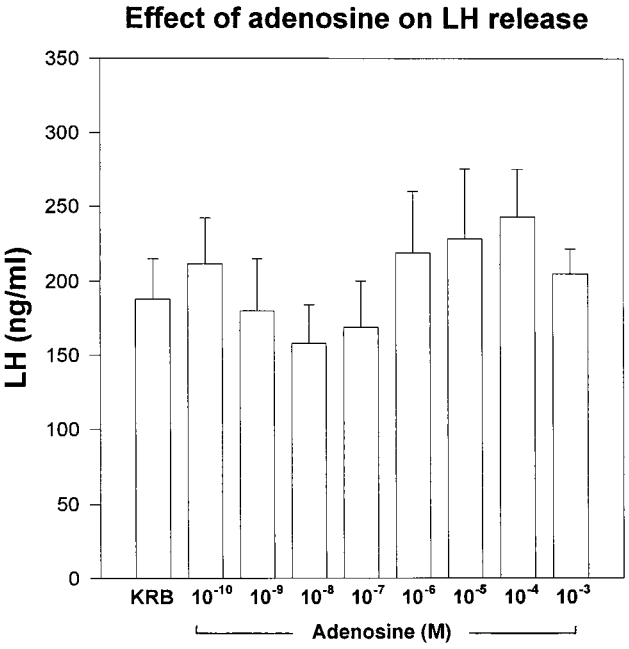

FIG. 3. The effect of adenosine on LH release.

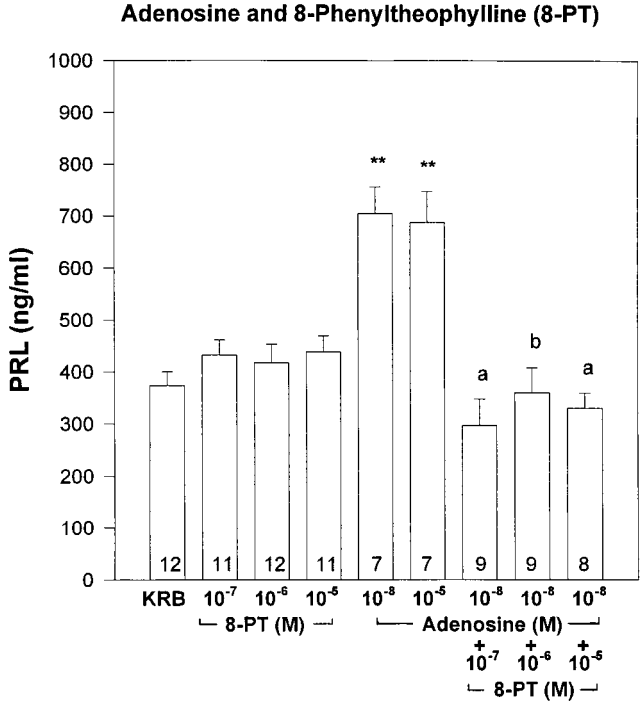

FIG. 4. The effect of adenosine and the A_1 receptor stimulator 8-phenyltheophylline (8-PT) on PRL release. a, $P < 0.001$ and b, $P < 0.01$ vs. the 10^{-8} M adenosine group. The numbers at the base of the columns = nos. of pituitaries per group.

curve of FSH release that was significant at the adenosine concentration (10^{-8} M) that maximally stimulated PRL secretion.

Since two highly selective A_1 adenosine receptor antagonists, 8-phenyltheophylline and 8-cyclopentyltheophylline (6),

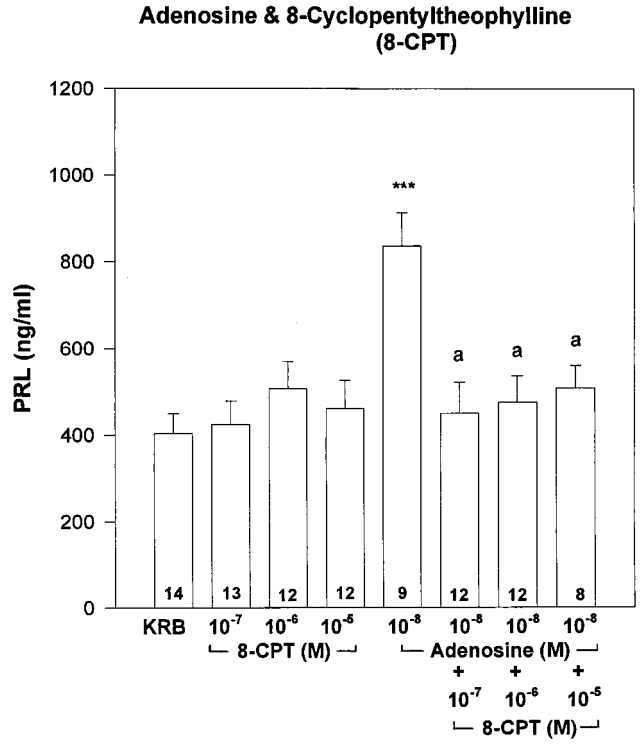

FIG. 5. The effect of adenosine and another A_1 receptor-stimulating drug, 8-cyclopentyltheophylline (8-CPT), on PRL release. a, $P < 0.01$ vs. 10^{-8} M adenosine group.

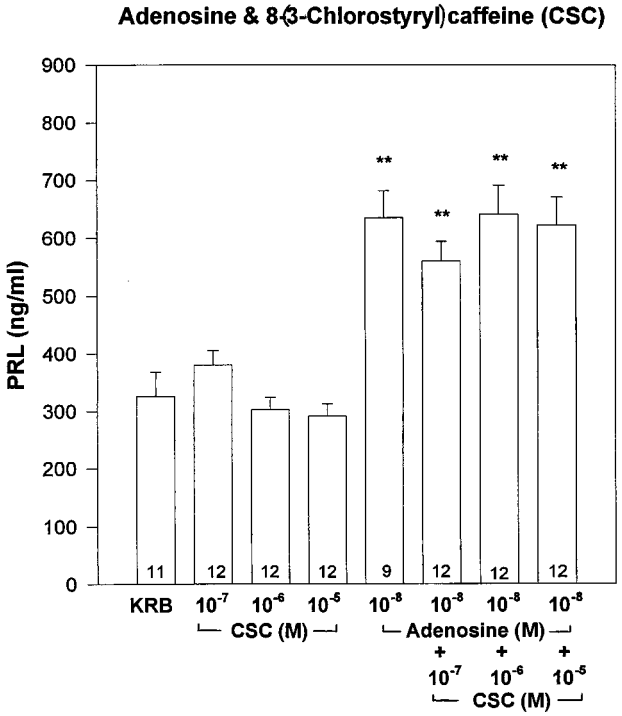

FIG. 6. The effect of adenosine and an A_{2a} receptor stimulator, 8-(3-chlorostyryl) caffeine (CSC), on PRL secretion. **, $P < 0.01$ versus KRB controls.

completely suppressed adenosine-induced PRL release, whereas 8-(3-chlorostyryl) caffeine, a selective A_{2a} adenosine receptor antagonist (6), had no effect on the stimulation induced by adenosine, the results indicate that adenosine acts on A_1 but not A_{2a} receptors in the anterior pituitary to stimulate the release of PRL. Since the A_1 and A_2 receptor blockers also did not alter PRL release by themselves, it appears that adenosine plays no role in basal PRL release *in vitro*.

The simplest explanation of the results is to postulate that the adenosine receptors are located on the lactotrophs; however, the evidence indicates that adenosine is secreted by the FS cells in the pituitary gland (3, 4). These cells also produce and secrete cytokines such as interleukin 6 (9) and interleukin 1β (10). They also contain neural nitric oxide synthase (NOS)

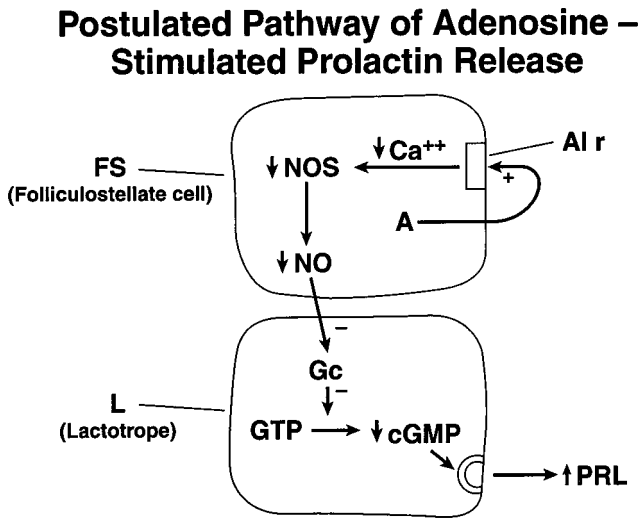

FIG. 7. Postulated pathway of adenosine-stimulated PRL release. For details, see the text.

(11). Indeed, nitric oxide (NO) has an inhibitory effect on PRL release mediated by cGMP (12), and, furthermore, it has been shown that the inhibitory action of dopamine mediated by dopamine$_2$ (D_2) receptors, known to be located on FS cells, is mediated by activation of NOS and release of NO that diffuses into the lactotrophs (12). There NO activates guanylate cyclase that generates cGMP from GTP. The cGMP activates protein kinase G that inhibits exocytosis of PRL secretary granules (12). We hypothesize that adenosine secreted by the FS cells acts on A_1 receptors on these cells to inhibit NOS, thereby reducing the secretion of NO. This reduction in NO reaching the lactotrophs and consequent decreased cGMP release would result in a stimulation of PRL release (Fig. 7).

Indeed, A_1 receptors have been shown to activate inhibitory G proteins (Gi) that decrease intracellular calcium concentrations (13). Since an increase in intracellular calcium is required to activate neural NOS (12) in the FS cells, the decrease in intracellular calcium induced by the activation of the A_1 receptors by adenosine should decrease the activity of NOS, thereby decreasing NO release, decreasing its diffusion to the lactotrophs. The resultant decreased cGMP generation in the lactotrophs causes an increase in PRL secretion.

This mechanism of action of the A_1 receptors to decrease intracellular calcium, if A_1 receptors are located on the cell membrane of the lactotrophs, should decrease rather than increase PRL release (14). Therefore, we postulate that the A_1 receptors are located on the FS cells and not on the lactotrophs themselves. Additional experiments are required to differentiate between these two possibilities, including studies to identify which pituitary cell types express the A_1 receptor and NOS.

An effect of adenosine on PRL secretion was first demonstrated by Ondo *et al*. (15), who reported that injection of adenosine into the third cerebral ventricle elevated plasma concentrations of PRL in the rat. It was postulated that the action of adenosine was exerted within the hypothalamus; however, it is possible that sufficient adenosine entered the hypophysial portal vessels to have a direct effect on the pituitary gland.

On the other hand, adenosine inhibited PRL release in cultures of anterior pituitary cells (16). These opposite results to ours could be explained on the basis of loss of paracrine actions of NO in cultures of anterior pituitary cells. In that circumstance because of the greater diffusion distance between cultured cells as opposed to cells in the undisrupted gland, if adenosine were acting on the FS cells to reduce the release of NO, in all probability, the concentration of NO reaching the lactotrophs would already be so small that adenosine would produce no significant reduction in NO concentration in the lactotrophs. Therefore, PRL release would not increase. In fact, the results of Schettini *et al*. (16) suggest that in cultured cells with a postulated loss of the paracrine action of NO, there may actually be A_1 receptors on the lactotrophs, which, as would be expected by the ability of the A_1 receptors to lower intracellular calcium concentrations, would lead to a reduction in PRL release as they reported instead of the increase seen here.

In striking contrast to the stimulation of PRL release induced by adenosine, it had an opposite action to inhibit FSH release with a dose-response relationship that was the mirror image of that for PRL. We have shown that FSH release induced by LH-releasing hormone, FSHRF, and leptin is controlled by NO via cGMP release (17). Therefore, we hypothesize that the reduction in NO release from the FS cells induced by adenosine would result in decreased concentrations of NO and consequently cGMP released in FSH gonadotrophs with resultant decreased FSH release.

Since LH release evoked by LH-releasing hormone and leptin is also controlled by NO-induced cGMP release, the failure of LH release to be significantly inhibited by adenosine is surprising. This failure could be explained if the diffusion distance to the LH gonadotrophs of NO released from the FS cells was greater than that to the FSH gonadotrophs.

Since adenosine has been shown to be secreted by pituitary cell cultures (J. C. Porter, unpublished data) and in view of its remarkable capability to stimulate PRL release, we hypothesize that adenosine plays a physiological role in control of PRL release. Since adenosine plays a physiologically significant role in the heart to dilate the coronary arteries (18), it may play a similar role to dilate the hypophysial portal vessels, thereby increasing anterior pituitary blood flow. Additional studies are necessary to determine the stimuli that release adenosine in the pituitary and its physiological or pathological significance *in vivo*.

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