

Mechanism of D₂ Agonist-induced Inhibition of GH Secretion from Human GH-secreting Adenoma Cells

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Abstract. The mechanism of dopamine D₂ agonist-induced inhibition of GH secretion from GH-secreting adenoma cells was investigated by measurement of intracellular calcium concentration ($[Ca^{2+}]_i$) and static incubation experiment. Bromocriptine decreased $[Ca^{2+}]_i$ in a concentration-dependent manner through D₂ receptor. The inhibition was abolished by pertussis toxin pretreatment. Bromocriptine did not decrease $[Ca^{2+}]_i$ after nitrendipine had decreased it. 8Br-cAMP increased $[Ca^{2+}]_i$ but application of bromocriptine decreased it, suggesting that bromocriptine-induced inhibition of $[Ca^{2+}]_i$ is not dependent on bromocriptine-induced inhibition of adenylyl cyclase. Static incubation experiment revealed that bromocriptine inhibited GH secretion in a concentration-dependent manner. The inhibition was through D₂ receptor and was abolished by pertussis toxin pretreatment. 8Br-cAMP increased GH secretion. Bromocriptine decreased GH secretion even after 8Br-cAMP pretreatment. However, the GH release from cells incubated with bromocriptine alone was significantly less than that from cells incubated with bromocriptine after 8Br-cAMP pretreatment, suggesting a modulatory action of cAMP system in bromocriptine response.

Key words: Acromegaly, D₂ receptor, GH secretion, Intracellular calcium concentration, cAMP

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DOPAMINE type 2 receptor agonists (D₂ agonists) are used to clinically treat acromegalic patients [1]. Oral bromocriptine (a D₂ agonist) treatment decrease serum GH concentration in about half of the patients. However, the mechanisms by which D₂ agonists inhibit GH secretion in GH-secreting adenomas are not fully elucidated. It is well known that D₂ receptor is coupled to Gi protein that inhibits adenylyl cyclase, thereby decreasing intracellular cAMP concentration [2]. This mechanism has been proposed to be involved in the D₂ agonist-induced inhibition of GH secretion [3]. On the other hand, it is known in prolactin-secreting cells that D₂ agonists inhibit prolactin secretion by decreasing intracellular calcium concentration [4]. Therefore it

is also possible that a similar mechanism is involved in the inhibition of GH secretion from GH-secreting adenomas by D₂ agonists. Whether and how these two mechanisms are involved in the paradoxical inhibition of GH secretion in GH-secreting adenoma cells have yet to be investigated. In order to answer this question, we measured intracellular calcium concentration by fura 2-based method and determined the GH secretion from the adenoma cells.

Materials and Methods

Drugs

Dopamine, bromocriptine, nitrendipine, and sulpiride were purchased from Sigma (St. Louis, MO, USA). Dispace was purchased from Godo Shusei Co., Ltd. (Tokyo, Japan) and Fura 2/AM from Molecular Probes (Eugene, OR).

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Cell preparation

GH-secreting pituitary adenomas were obtained with informed consent from two patients operated for GH-secreting pituitary adenoma by transsphenoidal surgery (patient 1 and 2). This study and the use of human pituitary tissues resected at surgery as experimental materials is permitted by the Ethical Committee of University of Tokyo Faculty of Medicine. Preoperative serum GH levels of these patients were 22.4 ng/mL in patient 1 and 12.2 ng/mL in patient 2. Both of these patients showed paradoxical GH decrease by oral bromocriptine administration test. Nadir GH value after bromocriptine administration was 3.5 ng/mL in patient 1 and 2.0 ng/mL in patient 2. The adenoma tissues were minced into small pieces (<1 mm) and were digested with 1000 U/ml dispase. For investigating the hormonal release, cells were seeded on 24-well dishes at a density of 1×10^5 cells/well. For $[Ca^{2+}]_i$ measurements, cells were seeded on 22 mm round cover glasses and placed in 35 mm culture dishes. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), and kept in humidified air containing 5% CO₂ at 37°C. All experiments were carried out using cells cultured for 1 to 2 weeks.

$[Ca^{2+}]_i$ measurement

Cells were loaded with fura 2 by incubating with 2 μ mol/L fura 2/AM in Hanks' balanced salt solution containing 0.1% bovine serum albumin (BSA) for 40 min at room temperature. $[Ca^{2+}]_i$ measurements were performed on a Nikon Diaphot microscope (Nikon, Tokyo, Japan). Each cell was excited at 340 nm and 380 nm alternately at a frequency of 100 Hz with CAM220 (Jasco, Tokyo, Japan). A band filter was used to monitor the fluorescent emission at 510 nm. The cytosolic free Ca²⁺ concentration was determined from the equation $[Ca^{2+}]_i = K(R - R_{min})/(R_{max} - R)$ [5]. In this equation, K represents $K_d(F_{min}/F_{max})$, where K_d is the dissociation constant of fura 2 (130 nM at 25°C), and F_{max}/F_{min} is the ratio of Ca²⁺-free and Ca²⁺-bound fura 2 fluorescence at 380 nm. R_{min} is the 340/380 fluorescence ratio of Ca²⁺-free fura 2, and R_{max} is the 340/380 ratio of Ca²⁺-bound fura 2. Calibration was performed on every cell by permeabilizing the cell to Ca²⁺ with 2 μ M digitonin. Cells were first permeabilized in Ca²⁺-free saline (5 mM EGTA, 150 mM KCl,

and 10 mM HEPES, pH 7.2), for determination of R_{min} and F_{min} , and then in high Ca²⁺ saline (2.5 mM CaCl₂, 150 mM KCl, and 10 mM HEPES, pH 7.4) for determination of R_{max} and F_{max} . The $[Ca^{2+}]_i$ traces shown in the figures were filtered with a bandwidth of 1 Hz in order to reduce the noise. The standard external solution was as follows (in mmol/L): 128 NaCl, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, and 10 HEPES (Na salt, pH 7.4). A peristaltic pump was used to perfuse the external solution during the experiments. Agonists were applied by changing the perfusing solution. It took about 30 sec to change the bath solution in this system.

Static incubation experiment

Adenoma cells cultured in 24-well dishes were washed twice with serum-free DMEM containing 0.1% BSA. They were incubated with the same medium containing 1 μ mol/L bromocriptine for 2 hr with or without 1 μ mol/L nitrendipine. Some cells were pre-treated with pertussis toxin (PTX, 0.1 μ g/ml for 12 hr). After PTX pretreatment, cells were treated with or without 1 μ mol/L bromocriptine for 2 hr. After the incubation with various agents, the solution was collected and stored at -20°C until the hormonal assay. GH was assayed using a radioimmunoassay kit (Daiichi Radioisotope Laboratories, Tokyo, Japan).

Cell identification

At an early stage of the experiments, the cells were stained against human GH immunocytochemically to identify GH-secreting adenoma cells. Adenoma cells ($n = 7$) that showed $[Ca^{2+}]_i$ decrease to bromocriptine application were fixed by 2% formaldehyde in phosphate buffer and stained for human GH by using a human GH immunostaining kit (DAKO Co., Ltd., Glostrup, Denmark). All these cells stained positive for GH. In the subsequent experiments, data were obtained from cells which satisfied the criteria for pituitary adenoma cells obtained from our experience, which are round shaped cells with a diameter of 10–20 μ m which have a smooth glittering surface under scanning light microscope. These characteristics were apparently different from those of the spindle-shaped fibroblast-like cells that grew in the adenoma cell culture.

Results

D_2 agonist-induced decrease of $[Ca^{2+}]_i$

The effect of bromocriptine on the $[Ca^{2+}]_i$ was investigated by fura 2-based method. Fig. 1A shows the effect of bromocriptine (1 μ mol/L) on the intracellular calcium concentration from a cell from adenoma 1. Bromocriptine decreased calcium concentration in a reversible manner. The involvement of D_2 receptor was confirmed by a D_2 receptor antagonist sulpiride. After pretreatment with sulpiride (10 μ mol/L for 5 min), bromocriptine (1 μ mol/L) together with sulpiride failed to decrease calcium concentration (Fig. 1B). Similar results were obtained in 8 cells from adenoma 1 and 5 cells from adenoma 2. Fig. 1C summarizes the results of these data. The decrease in intracellular calcium concentration by bromocriptine at several concentrations was measured in 6 cells from adenoma 1. There was a significant concentration-dependent decrease of intracellular calcium concentration, and sulpiride abolished the bromocriptine effect.

When the cells were pretreated with pertussis toxin (0.1 μ g/ml for 12 hr), the effect of bromocriptine (1 μ mol/L) was abolished (Fig. 2A), indicating that a pertussis toxin-sensitive G protein is involved in the response. Similar results were obtained in 5 cells from adenoma 1 and 5 cells from adenoma 2. When nitrendipine (1 μ mol/L), an L-type voltage-gated calcium channel blocker, was applied to the cell from adenoma 1, intracellular calcium concentration was decreased.

Additional application of bromocriptine (1 μ mol/L) did not further decrease the calcium concentration (Fig. 2B), indicating that inhibition of L-type voltage-gated channel is responsible for bromocriptine-induced $[Ca^{2+}]_i$ decrease. Similar results were obtained in 5 cells from adenoma 1 and 5 cells from adenoma 2. To examine the involvement of inhibition of adenylyl cyclase by D_2 agonist in the regulation of intracellular calcium concentration, a cell permeable cAMP analogue, 8Br-cAMP was used. Application of 8Br-cAMP (100 μ mol/L) increased the calcium concentration (Fig. 2C). After the application of 8Br-cAMP, bromocriptine (1 μ mol/L) decreased intracellular calcium concentration to less than the control level. Similar results were obtained in 5 cells from adenoma 1 and 5 cells from adenoma 2. These data indicate that bromocriptine-induced calcium decrease was not dependent on the inhibition of adenylyl cyclase. A summary of these experiments is plotted in Fig. 2D.

D_2 agonist-induced inhibition of GH secretion

The effect of bromocriptine on GH secretion was investigated by static incubation experiment. The results are plotted in Fig. 3. Bromocriptine inhibited GH secretion in a concentration-dependent manner. The inhibition was abolished by co-incubation with a D_2 receptor antagonist, sulpiride (10 μ mol/L). The inhibitory effect was abolished by pretreating the cells with pertussis toxin (0.1 μ g/ml for 12 hr). Incubation of the cells with nitrendipine decreased GH secretion and

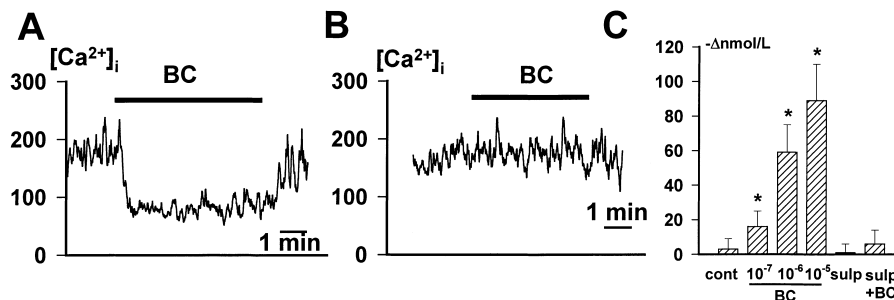


Fig. 1. Bromocriptine-induced $[Ca^{2+}]_i$ response in GH-secreting adenoma cells. Application of 1 μ mol/L bromocriptine decreased $[Ca^{2+}]_i$ (A). Pretreatment with sulpiride (10 μ mol/L) abolished bromocriptine response (B). A summary of these experiments is plotted in (C). The decrease in the $[Ca^{2+}]_i$ by bromocriptine ($-\Delta$ nmol/L) is plotted ($n = 6$ in each experiment). Data are shown as mean \pm S.D. Bars indicate 1 S.D. (* $P < 0.05$, compared to control) The data were analyzed by one-way ANOVA, which turned out to be significantly different ($p < 0.01$). The difference between each pair was analyzed by post-test (Tukey). There was a significant linear trend ($p < 0.01$) in $[Ca^{2+}]_i$ decrease by increasing doses of bromocriptine with post-test for linear trend. "cont" indicates $[Ca^{2+}]_i$ change in control medium without bromocriptine application, "sulp" that by application of control medium after sulpiride pretreatment, "sulp + BC" that by bromocriptine (1 μ mol/L) after sulpiride pretreatment, and "BC" that by bromocriptine application.

acromegalic patients. Our study revealed that the mechanisms of this paradoxical response involve D₂ agonist-induced decrease in intracellular calcium concentration.

D₂ receptor is known to couple to Gi protein that inhibits adenylyl cyclase. The D₂ agonists-induced inhibition of GH secretion has been attributed to the inhibition of adenylyl cyclase [3]. In our previous study, we examined the effect of dopamine on the membrane excitability of human GH-secreting adenoma cells that responded to D₂ agonists [7]. These adenoma cells elicited action potential firings at rest and dopamine hyperpolarized the membrane and shut down these action potential firings through D₂ receptor. We speculated from these data that dopamine decrease intracellular calcium concentration that is regulated by the calcium influx through the voltage-gated calcium channels. In this study, we proved the speculation is correct and further found that this calcium response was independent of D₂ agonist-induced inhibition of

adenylyl cyclase. In the previous study, we also proved that addition of high concentration of cAMP in the patch pipette solution in the whole cell mode did not abolish D₂ agonist-induced hyperpolarization. Therefore, the major mechanism of D₂ agonist-induced inhibition of GH secretion is the inhibition of excitability and resulting decrease in intracellular calcium concentration. However, it was also evident that cAMP system may modulate this D₂ agonist response because the bromocriptine-induced decrease in calcium concentration after 8Br-cAMP treatment was significantly less than that of control (Fig. 2D, column 1 and 4) and GH release with bromocriptine was significantly less than that by bromocriptine after 8Br-cAMP pretreatment. Although the mode of modulation of 8Br-cAMP on D₂ agonist action was not elucidated, it may affect the calcium channel or potassium channel that is responsible for D₂-induced hyperpolarization. Electrophysiological studies may provide further information.

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