Effect of colchicine and taxol on stimulation of G protein GTPase activity in anterior pituitary lobe of rats by gonadotrophin- and thyrotrophin-releasing hormones

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Colchicine and taxol, which are known to influence tubulin function, were used to delineate the possible role of tubulin in signal transduction in the anterior pituitary lobe. Anterior pituitary lobes, obtained from adult male rats, were processed by discontinuous sucrose gradient centrifugation to obtain plasma membranes. The low $K_m$ GTPase activity (EC 3.6.1.1) was assayed in 5 μg membrane protein using [$γ$-³²P]GTP at 37°C in an ATP-regenerating buffer containing 1 μmol unlabelled GTP l⁻¹. Ten nmol l⁻¹ each of colchicine, lumicolchicine and taxol maximally stimulated the GTPase activity by about 40% ($P < 0.05$). A time-course study revealed that 100 nmol colchicine l⁻¹ stimulated the enzyme activity by 55, 74, 89 and 53% at 5, 10, 20 and 30 min, respectively ($P < 0.05$); lumicolchicine (100 nmol l⁻¹) stimulated the GTPase activity by 44, 36, 11 and 55% at 5, 10, 20 and 30 min, respectively ($P < 0.05$). Taxol (100 nmol l⁻¹) stimulated the enzyme activity by 39 and 25% at 20 and 30 min, respectively ($P < 0.05$).

Gonadotrophin-releasing hormone (GnRH) and thyrotrophin-releasing hormone (TRH) stimulated the low $K_m$ GTPase activity in a concentration-dependent manner, by up to 40–60% ($P < 0.05$). In the presence of 100 nmol colchicine l⁻¹, the ability of GnRH or TRH to stimulate the GTPase activity was inhibited. For example, at 1 nmol GnRH l⁻¹, the enzyme activity was stimulated from 124 to 176 pmol min⁻¹ mg⁻¹ protein; in the presence of 100 nmol colchicine l⁻¹, activity stimulated by GnRH (1 nmol l⁻¹) was only 157 pmol min⁻¹ mg⁻¹ protein ($P < 0.05$). At 10 nmol TRH l⁻¹ the enzyme activity was stimulated from 124 to 174 pmol min⁻¹ mg⁻¹ protein; in the presence of 100 nmol colchicine l⁻¹, activity stimulated by TRH (10 nmol l⁻¹) was only 155 pmol min⁻¹ mg⁻¹ protein ($P < 0.05$). GnRH or TRH stimulation of the enzyme activity was not affected in the presence of lumicolchicine. In the presence of taxol, the stimulation of the GTPase activity by either GnRH or TRH was inhibited. GnRH (1 nmol l⁻¹) stimulated the GTPase activity from 124 to 150 pmol min⁻¹ mg⁻¹ protein; in the presence of 100 nmol taxol l⁻¹, activity stimulated by GnRH (1 nmol l⁻¹) was only 128 pmol min⁻¹ mg⁻¹ protein ($P < 0.05$); 1 nmol TRH l⁻¹ stimulated the GTPase activity from 124 to 174 pmol min⁻¹ mg⁻¹ protein; in the presence of 100 nmol taxol l⁻¹, activity stimulated by TRH (1 nmol l⁻¹) was only 157 pmol min⁻¹ mg⁻¹ protein ($P < 0.05$). These results indicate that these drugs inhibit hormone-stimulated G protein GTPase activity as a result of their interaction with tubulin or G protein(s) or both.

Introduction

Guanine-nucleotide-dependent regulatory proteins (G proteins) are a family of closely related proteins involved in the transfer of information from surface receptors to biochemical effector mechanisms in a variety of systems (Gilman, 1987; Birnbaumer, 1990; Birnbaumer et al., 1990; Boege et al., 1991; Dohlman et al., 1991; Kaziro et al., 1991; Strosberg, 1991). G proteins are

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of S49 mouse lymphoma cells (Carlson et al., 1986) have been observed. Signal transduction in neutrophils is coupled to actin polymerization (Omann et al., 1987; Therrien and Naccache, 1989; Bengtsson et al., 1990). Extraction of main classes of G protein with octyl glucoside resulted in large, polydisperse structures sensitive to disaggregation by GTP[yS], leading to the suggestion that these large structures facilitate the interaction of G proteins with the plasma membrane and the cytoskeletal matrix (Nakamura and Rodbell, 1990). The binding of tubulin to a G-protein affinity column (Higashi and Ishibashi, 1985) and to Gsa and Gna has been reported (Wang et al., 1990). Anti-tubulin antibodies, especially anti-β-tubulin antibody, stimulated the low K_m GTPase activity associated with G proteins in rat striatal membranes and potentiated the ability of acetylcholine to stimulate this activity, suggesting that tubulin acts as a GTP sink and exerts a chronic inhibitory influence on G protein function and receptor–G–protein interactions (Ravindra and Aronstam, 1990a). Colchicine, a drug known to inhibit tubulin polymerization, inhibited the stimulation of G protein GTPase activity by acetylcholine in rat striatal membranes, suggesting a role for tubulin in signal transduction (Ravindra and Aronstam, 1991).

In an attempt to understand further the role of tubulin in signal transduction processes, we examined the effects of colchicine (inhibits tubulin polymerization), lumicolchicine (does not affect tubulin polymerization) and taxol (promotes tubulin polymerization) on the low K_m GTPase activity associated with transducer G proteins in rat anterior pituitary lobes, including its stimulation by activation of gonadotrophin-releasing hormone (GnRH) and thyrotrophin-releasing hormone (TRH) receptors.

Materials and Methods

Materials

GnRH agonist (p Glu-His-Trp-Ser-Tyr-d-Ala-Leu-Arg-Pro-Gly-NH2), (catalog no. L-0512), colchicine (catalog no. C-9754) and β-lumicolchicine (catalog no. L-0635) were purchased from Sigma Chemical Co. (St Louis, MO). Taxol was a gift from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Radio-labelled guanosine-5'-triphosphate ([γ-32P]GTP; 1500 Ci mmol⁻¹) was purchased from ICN Radiochemicals (Irvine, CA). All other chemicals used in the study were purchased from Sigma.

Anterior pituitary membrane preparation

Plasma membranes were prepared according to the method of Hubbard et al. (1983), with some modifications. Adult, male Wistar rats (125–150 g, Harlan Sprague–Dawley, Indianapolis, IN) were housed in pairs in an environmentally controlled room with free access to food and water. Rats were killed by decapitation and their anterior pituitary lobes were removed. All the subsequent procedures were conducted at 4°C. Routinely, tissue from 30–40 rats was homogenized in buffer A (5 mmol Tris–HCl 1⁻¹ with 1 mmol EDTA 1⁻¹, 1 mmol dithiothreitol 1⁻¹, pH 7.4) using a motor-driven Teflon–glass tissue grinder. The homogenate was centrifuged at 800 g for 20 min. The supernatant was maintained on ice while the pellet was resuspended in buffer A and centrifuged at 800 g for 20 min. The combined supernatants were then centrifuged at 100 000 g for 60 min. The resulting pellet was resuspended in buffer A containing 0.7 mol sucrose 1⁻¹ and overlaid on a discontinuous sucrose gradient and spun at 60 000 g for 60 min; the gradient consisted of 4.5 ml of 1.3 mol sucrose-buffer A 1⁻¹, 2.5 ml of 1.0 mol sucrose-buffer A 1⁻¹ and 2.5 ml of sample. After centrifugation, 2.5 ml from the top of each tube was carefully collected, pooled, washed with buffer A and stored frozen at −80°C. Protein content was estimated according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

GTPase assay

Low K_m GTPase activity was determined in triplicate as described previously (Ravindra and Aronstam, 1990b). The reaction mixture (100 µl) contained 75 mmol Tris–HCl 1⁻¹ (pH 7.4), [γ-32P]GTP (70–100 000 c.p.m.), 1 µmol unlabelled GTP 1⁻¹, 2 mmol MgCl₂ 1⁻¹, 0.5 mmol ATP 1⁻¹, 0.5 mmol adenylylimidodiphosphate 1⁻¹, 5 mmol phosphocreatine 1⁻¹, creatine phosphokinase (50 units ml⁻¹), bovine serum albumin (50 µg), 0.1 mmol EDTA 1⁻¹, 0.2 mmol EGTA 1⁻¹, 1 mmol cAMP 1⁻¹, 100 mmol NaCl 1⁻¹ and 5 µg protein. All these components were added to 12 mm × 75 mm glass tubes on ice and the reaction was initiated by immersing the tubes in a 37°C water bath. After 20 min, the reaction was stopped by transferring the tubes to an ice bath followed immediately by the addition of 5% activated charcoal in 20 mmol phosphoric acid 1⁻¹ (pH 2.5). Samples were kept on ice for 10 min and centrifuged at 700 g for 10 min. An aliquot (100 µl) from the supernatant was mixed with 5 ml of Scintiverse BD (Fisher Scientific, Pittsburgh, PA) and the radioactivity content determined using a Beckman (Palo Alto, CA) liquid scintillation counter. Low K_m GTPase activity (EC 3.6.1.) was routinely calculated by subtracting activity measured in the presence of 100 µmol unlabelled GTP 1⁻¹ from total activity.

Statistical analysis

Statistical differences among various groups were determined by analysis of variance and Fisher tests using the STATVIEW II program on a Macintosh II computer.

Results

Effects of colchicine, lumicolchicine, and taxol on low K_m GTPase activity

In addition to its effect on tubulin function, colchicine is a potent inhibitor of nucleoside transport and aldose reductase, and stimulates DNA synthesis in human lymphocyte cultures and in fibroblast cultures of chick, mouse and human. In rats, colchicine increases adenylyl cyclase activity, prostaglandin E₂, and cAMP concentrations; colchicine also modifies the behaviour of membrane proteins (see, Ravindra and Grosvenor, 1990a). One way to examine the specificity of the colchicine effect is to use lumicolchicine, an isomer of colchicine that does not inhibit tubulin polymerization (Borisy et al., 1972).
We therefore investigated the effects of colchicine as well as lumicolchicine on low \( K_m \) GTPase activity: 10 nmol \( 1^{-1} \) each of colchicine, lumicolchicine and taxol maximally stimulated the GTPase activity by about 40% \( (P < 0.05; \text{Fig. 1a, b and c}) \). A time-course study revealed that 100 nmol colchicine \( 1^{-1} \) stimulated the enzyme activity by 55, 74, 89 and 53% at 5, 10, 20 and 30 min, respectively \( (P < 0.05; \text{Fig. 2a}) \); lumicolchicine (100 nmol \( 1^{-1} \)) stimulated the GTPase activity by 44, 36, 11 and 55% at 5, 10, 20 and 30 min, respectively \( (P < 0.05; \text{Fig. 2b}) \). Taxol (100 nmol \( 1^{-1} \)) stimulated the enzyme activity by 39 and 25% at 20 and 30 min, respectively \( (P < 0.05; \text{Fig. 2c}) \).

**Influence of colchicine, lumicolchicine, and taxol on GnRH or TRH stimulation of low \( K_m \) GTPase activity**

GnRH and TRH stimulated the low \( K_m \) GTPase activity in a concentration-dependent manner, by 40–60% \( (P < 0.05; \text{Figs 3 and 4}) \). In the presence of 100 nmol colchicine \( 1^{-1} \), the ability of GnRH or TRH to stimulate the GTPase activity was inhibited. For example, at 1 nmol GnRH \( 1^{-1} \), the enzyme activity was stimulated from 124 to 176 pmol min \( ^{-1} \) mg \( ^{-1} \) protein; in the presence of 100 nmol colchicine \( 1^{-1} \), activity stimulated by GnRH (1 nmol \( 1^{-1} \)) was only 157 pmol min \( ^{-1} \) mg \( ^{-1} \) protein \( (P < 0.05; \text{Fig. 3a}) \). At 10 nmol TRH \( 1^{-1} \), the enzyme activity was stimulated from 124 to 174 pmol min \( ^{-1} \) mg \( ^{-1} \) protein; in the presence of 100 nmol colchicine \( 1^{-1} \), activity stimulated by TRH (10 nmol \( 1^{-1} \)) was only 155 pmol min \( ^{-1} \) mg \( ^{-1} \) protein \( (P < 0.05; \text{Fig. 3b}) \). GnRH or TRH stimulation of the enzyme activity was not affected in the presence of lumicolchicine. In the presence of taxol, the stimulation of the GTPase activity by either GnRH or TRH was inhibited: 1 nmol GnRH \( 1^{-1} \) stimulated the GTPase activity from 124 to 150 pmol min \( ^{-1} \) mg \( ^{-1} \) protein; in the presence of 100 nmol taxol \( 1^{-1} \), activity stimulated by GnRH (1 nmol \( 1^{-1} \)) was only 128 pmol min \( ^{-1} \) mg \( ^{-1} \) protein \( (P < 0.05; \text{Fig. 4a}) \); 1 nmol \( 1^{-1} \) TRH stimulated the GTPase activity from 124 to 174 pmol min \( ^{-1} \) mg \( ^{-1} \) protein; in the presence of 100 nmol taxol \( 1^{-1} \), activity stimulated by TRH (1 nmol \( 1^{-1} \)) was only 157 pmol min \( ^{-1} \) mg \( ^{-1} \) protein \( (P < 0.05; \text{Fig. 4b}) \).

**Discussion**

Compounds that interact with tubulin have been used extensively by many investigators to understand the role of tubulin in cellular processes. Colchicine is considered to be a prototype of a class of chemical inhibitors that act on microtubules; colchicine inhibits the polymerization of tubulin monomers into microtubule polymers, finally resulting in the

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**Fig. 1.** Effect of (a) colchicine, (b) lumicolchicine and (c) taxol on low \( K_m \) GTPase activity. Rat anterior pituitary plasma membranes (5 µg) were incubated for 30 min with various concentrations of drugs and the enzyme activity determined at 1 µmol GTP \( 1^{-1} \). Each point represents the mean \( \pm SD \) of three determinations from one experiment. *Value significantly different from the control value \( (P < 0.05) \). This is a representative of three experiments.
Transducer G proteins and tubulin are both 'G proteins' in the sense that their activity is regulated by guanine nucleotides. Similarities between G proteins and tubulin include (i) considerable homology and a highly conserved nature (Sternlicht et al., 1987), (ii) ADP ribosylation sites for cholera and pertussis toxins (Lim et al., 1985) and (iii) Mg$^{2+}$-dependent binding and hydrolysis of GTP (Gilman, 1987; Carlier, 1989). The $K_m$ of GTPase activity associated with G proteins (EC 3.6.1.) is quite low. For example, 0.5–0.7 µmol l$^{-1}$ in rat striatum (Ravindra and Aronstam, 1990a), 0.1 µmol l$^{-1}$ in turkey erythrocytes (Cassel and Selinger, 1976) and 0.6 µmol l$^{-1}$ in rat anterior pituitary lobe (R. Ravindra and R. S. Aronstam, unpublished).

The low $K_m$ GTPase assay is conducted in an ATP-regenerating system and assay conditions are manipulated such that only 10–20% of added GTP is hydrolysed. The ATP-regenerating system inhibits GTP hydrolysis by nonspecific nucleosidases and the transfer of phosphate from GTP to ATP. On the other hand, the tubulin-GTPase activity is not a typical enzyme activity and hence a $K_m$ value for this reaction has not been determined (Carlier, 1989). Tubulin-GTPase activity is generally assayed using buffers suitable for tubulin polymerization (Ravindra and Grosvenor, 1990b).

The binding of an agonist to the receptor facilitates, in the presence of Mg$^{2+}$, an exchange of GTP for GDP on the α subunit. The activated α subunit dissociates from the βγ subunits, and one or both interact with effector molecules such as phospholipase C (Birnbaumer, 1990; Birnbaumer et al., 1990).

An intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP, releasing inorganic phosphate (Pi); α$_{GDP}$ then recombines with βγ, ending the activation cycle (Gilman, 1987). Stimulation by GnRH and TRH of GTP binding and GTPase activity (EC 3.6.1.) in the rat anterior pituitary lobe has recently been demonstrated (Ravindra and Aronstam, 1990b). In the present study, we investigated the effects of colchicine, lumicolchicine and taxol on GnRH-stimulated G protein GTPase activity, as this assay provides a simple and direct assessment of the receptor–G-protein interaction.

**Fig. 2.** Time course of low $K_m$ GTPase activity in the presence of (a) colchicine (control (○), colchicine (●)), (b) lumicolchicine (control (○), lumicolchicine (●)) and (c) taxol (control (○), taxol (●)). Rat anterior pituitary plasma membranes (5 µg) were incubated with 100 nmol colchicine, lumicolchicine, or taxol l$^{-1}$ and the enzyme activity was determined at the indicated time points. Each point represents the mean ± SD of three determinations from one experiment. *Value significantly different from control value (P < 0.05). This is a representative of two experiments. Where not shown, the error bar is smaller than the symbol representing the mean.
Fig. 3. Effect of colchicine or lumicolchicine on low $K_m$ GTPase activity stimulated by (a) gonadotrophin-releasing hormone (GnRH (○), GnRH + colchicine (●), GnRH + lumicolchicine (□)) and (b) thyrotrophin-releasing hormone (TRH (○), TRH + colchicine (●), TRH + lumicolchicine (□)). Rat anterior pituitary plasma membranes (5 µg) were incubated for 30 min with various concentrations of hormone in the presence or absence of 100 nmol colchicine or lumicolchicine l⁻¹ and the enzyme activity determined at 1 µmol GTP l⁻¹. Each point represents the mean ± SD of three determinations from one experiment. *Value significantly different from the activity obtained with hormone alone ($P < 0.05$). This is a representative of two experiments.

Fig. 4. Effect of taxol on low $K_m$ GTPase activity stimulated by (a) gonadotrophin-releasing hormone (GnRH (○), GnRH + taxol (●)) and (b) thyrotrophin-releasing hormone (TRH (○), TRH + taxol (●)). Rat anterior pituitary plasma membranes (5 µg) were incubated for 30 min with various concentrations of hormone in the presence or absence of 100 nmol taxol l⁻¹ and the enzyme activity determined at 1 µmol GTP l⁻¹. Each point represents the mean ± SD of three determinations from one experiment. *Value significantly different from the activity obtained with hormone alone ($P < 0.05$). This is a representative of two experiments.

It is important to note that not all of the low $K_m$ GTPase activity may be attributed to G proteins; there are other proteins in the anterior pituitary membranes that can hydrolyse GTP. In the present study, only hormone-stimulated activity was therefore considered to reflect activity of transducer G proteins. Stimulation of the basal low $K_m$ GTPase activity by colchicine, lumicolchicine and taxol indicates that these compounds may act directly on G proteins or on other GTP-binding proteins. To delineate the mechanism of action of these three drugs on G protein GTPase activity, it is necessary to study the effect of the drugs on purified G proteins.

Colchicine (which inhibits tubulin polymerization) inhibited the hormone-stimulated GTPase activity, and lumicolchicine (which does not inhibit tubulin polymerization) did not inhibit the hormone-stimulated GTPase activity, indicating that tubulin polymerization may be involved in pituitary signal transduction. However, the fact that both colchicine and taxol, which also have opposite effects on tubulin polymerization, inhibit the GnRH- and TRH-stimulated low $K_m$ GTPase activity suggests that tubulin polymerization, per se, is not involved in the action of the drugs. Structural proteins, such as tubulin, can participate in cellular signalling by communicating through physical forces
(Nakamura and Rodbell, 1990). By virtue of its interaction with the submembranous network of cytoskeletal proteins, tubulin, when perturbed in one locus, can transmit large changes in conformations to other points. Thus, colchicine (or taxol) binding to tubulin might lead to a conformational change in either the hormone receptor or G proteins. This may, in turn, lead to (i) inhibition of GnRH (or TRH) binding to its receptor, or (ii) an interference with GnRH (or TRH) receptor-G-protein coupling. However, the present data do not distinguish between these two possibilities.

In the presence of colchicine (100 nmol l⁻¹) hormone-stimulated GTPase activity was inhibited at 0.1–100 nmol GnRH l⁻¹ and 10–100 nmol TRH l⁻¹ (Fig. 3a, b), suggesting that GnRH receptor-G-protein interaction is more sensitive to colchicine action. Another aspect of this work that is not quite clear is the antagonistic action of the hormones on drug-stimulated GTPase activity. For example, 100 nmol colchicine l⁻¹ alone stimulated the enzymatic activity by up to 32%; in the presence of 0.1 nmol GnRH l⁻¹ and colchicine, the stimulation was only 15%. It appears that the presence of both GnRH (or TRH) and colchicine (or taxol) at the membrane level would reduce the affinity of GTP to G proteins, finally leading to reduced GTP hydrolysis. The physiological consequences of this intriguing phenomenon can be understood only when we know the identity of the G protein(s) involved and which of these G proteins are coupled to the GnRH (or TRH) receptor. It is established that G₁αG₂ and G₃ᵦ are present in the rat pituitary (Bouvier et al., 1991a, 1991b); the role of G₁ in the rat pituitary is not clear. Studies with rat pituitary GH₃ cells showed the involvement of G₀ in K⁺ channel activation (Birnbaumer et al., 1990) and that G₀ specifically mediated the carbabilch- and somatostatin-induced inhibition of voltage-sensitive Ca²⁺ channels (Kleuss et al., 1991). The second messengers inositol 1,4,5-trisphosphate (InsP₃) and 1,2-diacylglycerol are formed by the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP₂) by phospholipase C. Recently G₁₇, a novel G protein implicated in the regulation of phospholipase C, has been purified from bovine liver (Taylor et al., 1991) and brain (Smrcka et al., 1991). Since both GnRH and TRH stimulate phospholipase C (Birnbaumer et al., 1990), it is possible that G₁₇ is also present in the rat pituitary.

We have previously reported that 100 μmol colchicine l⁻¹ inhibited acetylcholine-stimulated G protein GTPase activity in rat striatal membranes (Ravindra and Aronstam, 1991); in the present study, 100 nmol colchicine l⁻¹ was sufficient to depress the hormone stimulation of the enzyme activity. This difference in the drug concentration might reflect tissue specificity or differences in experimental conditions. In fact, pure pituitary plasma membranes were used in the present study, whereas crude striatal membranes were used previously. In spite of the difference in the effective drug concentration, colchicine inhibited receptor-G-protein coupling in all three receptor systems.

We have demonstrated that colchicine, lumicolchicine and taxol stimulate the low Kₘ GTPase activity, but only colchicine and taxol block GnRH stimulation of the enzyme activity. These compounds may influence GnRH receptor-G-protein coupling as a consequence of their interaction with tubulin or G protein(s) or both.

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