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# Inhibitory Effect of Cyclosporin A on Prolactin Synthesis in $GH_3$ Cells

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Nagai, Y., Ohsawa, K., Ohta, M., Hisada, A., Yamashita, H., Yoshizawa, M., Yamamoto, Y., Takamura, T. and Kobayashi, K. *Inhibitory Effect of Cyclosporin A on Prolactin Synthesis in $GH_3$ Cells.* Tohoku J. Exp. Med., 1996, **180** (4), 337-346 —— Cyclosporin A (CsA), a potent immunosuppressant, is known to have various effects on the endocrine system, including the pituitary gland, the adrenal cortex, the testes, and the pancreatic islets. In this study, the effects of CsA on prolactin (PRL) synthesis and release were investigated in $GH_3$ cells, a clonal strain of rat pituitary tumor. After incubation of confluent $GH_3$ cells with various concentrations of CsA for 24 hr, the PRL content of the media decreased in a dose-dependent manner: by 28.5% with 100 ng/ml CsA ($p<0.01$); and 45.8% with 2,000 ng/ml CsA ($p<0.001$), compared with control. However, no significant change was observed in the intracellular PRL content. After removal of CsA from the medium, $GH_3$ cells fully recovered normal secretory activity within 24-48 hr, thus indicating that the inhibitory effect of CsA on PRL secretion was reversible. Northern blot analysis revealed a decrease in the PRL mRNA level in cells treated with CsA. In conclusion, these data suggest that CsA inhibits PRL secretion by reducing the rate of biosynthesis. A possible site of action is on PRL gene expression at the level of mRNA transcription. —— cyclosporin A; prolactin synthesis; prolactin mRNA; $GH_3$ cells

Cyclosporin A (CsA), a cyclic undecapeptide produced by the fungus *Tolypocladium inflatum*, is a potent immunosuppressant that is widely used to prevent rejection during organ transplantation and in the treatment of some autoimmune disorders (Borel et al. 1976; European Multicentre Trial Group 1982; Stiller et al. 1984). CsA exerts an immunosuppressive effect by inhibiting the transcriptional activation of interleukin 2 (IL-2) in helper T cells (Elliott et al. 1984; Kronke et al. 1984). Recently, CsA also has been shown to exert effects on the rat endocrine system, including suppression of androgen synthesis (Rajfer et al. 1987; Sikka et

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al. 1988), inhibition of adrenocortical function (Rebuffat et al. 1989), suppression of the secretion of adrenocorticotropin and corticosterone (Hirano et al. 1988) and inhibition of insulin secretion (Robertson 1986). The effect of CsA on the secretion of prolactin (PRL) remains controversial. Cardon et al. (1984) showed a rapid elevation in serum PRL concentrations following the intraperitoneal (i.p.) administration of CsA in rats. In contrast, Davenport and Hodson (1992) failed to observe any change in either the serum PRL concentration or pituitary PRL content following daily CsA administration (15 mg/kg per day i.p.) for 21 days. We recently reported an inhibitory effect of CsA on the secretion of PRL in patients with nephrotic syndrome (Nagai et al. 1992). However, since previous studies were performed in vivo, it is unclear whether or not CsA has a direct effect on the pituitary gland. In this study, the direct effect of CsA on PRL secretion was investigated in $GH_3$ cells, a clonal strain of rat pituitary tumor (Tashjian et al. 1968).

## Materials and Methods

### *Chemicals*

CsA was a gift from Sandoz Ltd. (Basel, Switzerland). Crystalline drug was first dissolved in absolute ethanol at a concentration of 10 mg/ml and then diluted to the appropriate concentrations by ethanol. [$\gamma$-$^{32}$P] ATP (3,000 Ci/mmol) was obtained from Amersham Japan Co. (Tokyo). Nonidet P40 and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were purchased from Wako Junyaku Co. (Osaka).

### *Cell culture*

$GH_3$ cells, a subclone of the tumor-derived rat pituitary cell line (Tashjian et al. 1968), were grown in monolayers in Ham's F-10 medium (GIBCO, New York, NY, USA) supplemented with 15% heat-inactivated horse serum (GIBCO), 2.5% fetal calf serum (GIBCO), penicillin (50 U/ml) and streptomycin (50 mg/ml) in a humidified atmosphere 95% air-5% $CO_2$. For determination of PRL secretion, cells were subcultured and plated on 12-well dishes at $2\times10^5$ cells/dish in 1 ml culture medium. For determination of PRL mRNA accumulation, $2\times10^5$ cells were plated per 25 cm$^2$ culture flask.

### *Effect of CsA on PRL secretion*

For CsA dose-response studies, the cells were incubated for 24 hr with 0-2,000 ng/ml CsA. At the end of the incubation period, the media were decanted, centrifuged at $3,000\times g$ for 15 min at 4°C (to remove dislodged cells), and stored at −20°C until assayed. Intracellular PRL was solubilized with Nonidet P40, as previously described (Walker and Farquhar 1980). Briefly, the cells were frozen, thawed, and then lysed in immunoprecipitation buffer containing 0.5% Nonidet P40. The cells were then scraped off the dish with a rubber policeman, pipetted

into test tubes, and sonicated for 10 sec using a sonifier cell disruptor. Finally, the pH was adjusted to 9 using 10 N NaOH, and the medium was allowed to stand at room temperature for 3 hr before readjustment to pH 7.4 for assay. For CsA time-response studies of the PRL secretion, cells were incubated with or without 100 ng/ml CsA for 4–48 hr and the PRL content in the media was determined at each timepoint. To investigate whether the effect of CsA on PRL secretion was reversible, the medium was removed after incubation with or without 100 ng/ml CsA for 24 hr, and the cells were washed twice in phosphate-buffered saline (PBS). The cells were then maintained in fresh medium without CsA, with exchanges every 24 hr until 72 hr. The PRL contents of the media were assayed at 24, 48 and 72 hr after the removal of CsA.

*PRL assay*

PRL content was measured using a double-antibody radioimmunoassay using Rat PRL [$^{123}$I] Assay System (Amersham Japan Co.) and expressed as ng/well. The sensitivity of the assay was 0.7 ng/ml. The coefficients of variation within and between assays were 3.2% and 8.0±0.1%, respectively.

*Northern blot analysis*

Two cell cultures were incubated either with or without CsA (100 ng/ml) for 24 hr. Total RNA was purified using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). RNA, either 10 or 20 $\mu$g, was denatured, separated on 1.5% agarose formaldehyde gel and blotted on BA85 nitrocellulose membrane (Schleicher & Schuell, New York, NY, USA). After baking for 2 hr at 80°C, the filter was prehybridized for 2 hr, and hybridized with a radioactive complementary DNA (cDNA) probe (10 ng/ml) for rat PRL in 5× SSPE, 50% formamide, 0.1% SDS, 1 mg/ml salmon sperm DNA, and 5×Denhardt's solution for more than 12 hr at 37°C. The filter was washed in 3×SSC, 0.1% SDS for 30 min, in 0.1×SSC, 0.1% SDS for 30 min at room temperature, and in 0.1×SSC, 0.1% SDS for 30 min at 37°C, and then exposed to Kodak x-ray film with an intensifying screen for 24 hr at −80°C. The length of the respective RNA was identified by staining the transfer with ethidium bromide and comparing the bands to standards of known length. The probe used in this study was a synthetic oligonucleotide 5′-GTAGTGAGAAAGCATGACCACA-CGGTCAAACAGCTCCGGGAG-3′ complementary to nucleotides 121–162 from the transcription initiation site of the rat PRL mRNA sequence (Cooke et al. 1980). The probe was labeled with [$\gamma$-$^{32}$P] ATP by 5′-end labeling method to a specific activity of approximately 1–2×10$^8$ cpm/$\mu$g DNA.

*Statistical analysis*

All values are expressed as the means±S.D. In comparing two groups, $p$ values were calculated by Student's $t$-test. In experiments involving compari-

sons of multiple groups, the probability that differences existed between the means of groups was determined by ANOVA using the least significant difference for multiple comparisons. A $p$ value of less than 0.05 was considered significant.

## Results

### *Effect of CsA on PRL secretion*

Exposure to CsA decreased the PRL content of the media in a dose-dependent manner: by 28.5% with 100 ng/ml CsA ($p<0.01$) and 48.5% with 2,000 ng/ml CsA ($p<0.001$), compared with control cells (Fig. 1). In contrast, the intracellular content of PRL following the administration of 1-50 ng/ml CsA appeared to be slightly increased compared with control cells, while the intracellular content of cells exposed to 500-2,000 ng/ml CsA appeared decreased. However, these changes were not statistically significant (Fig. 2). The time-course of the inhibitory effect of CsA on PRL secretion was 79.8% of controls at 12 hr ($p<0.05$), 71.5% of controls at 24 hr ($p<0.01$), 70.4% of controls at 36 hr ($p<0.05$), and 59.2% of controls at 48 hr ($p<0.05$) (Fig. 3). Next, we tested whether the inhibitory effect of CsA on PRL secretion was reversible. After removal of CsA from the medium, $GH_3$ cells fully recovered normal secretory activity within 24-48 hr (Fig. 4), thus indicating reversibility of the inhibitory effect of CsA on PRL secretion.

Cell viability after 24 hr incubation with or without CsA was determined by trypan blue exclusion. The cells were 94.6±2.9% viable without CsA, 93.1±2.4% viable with 100 ng/ml CsA and 91.2±1.8% viable with 2,000 ng/ml CsA

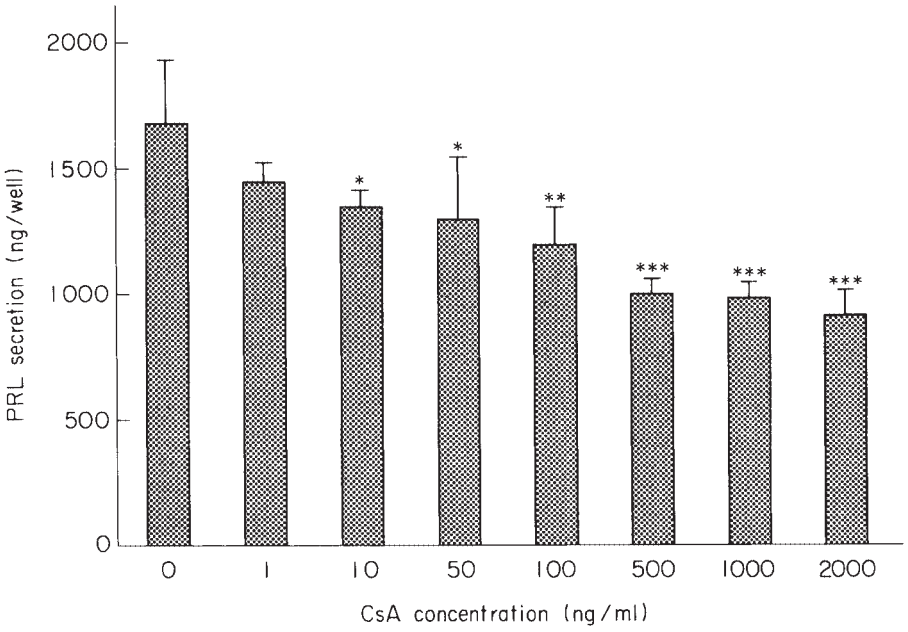


Fig. 1. Dose-dependent effect of cyclosporin A (CsA) on prolactin (PRL) secretion from $GH_3$ cells. After the incubation of confluent $GH_3$ cells with various concentrations of CsA for 24 hr, the PRL contents of the media (ng/well) were measured by radioimmunoassay (RIA). The values represent the mean±S.D. ($n=5$). $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with control.

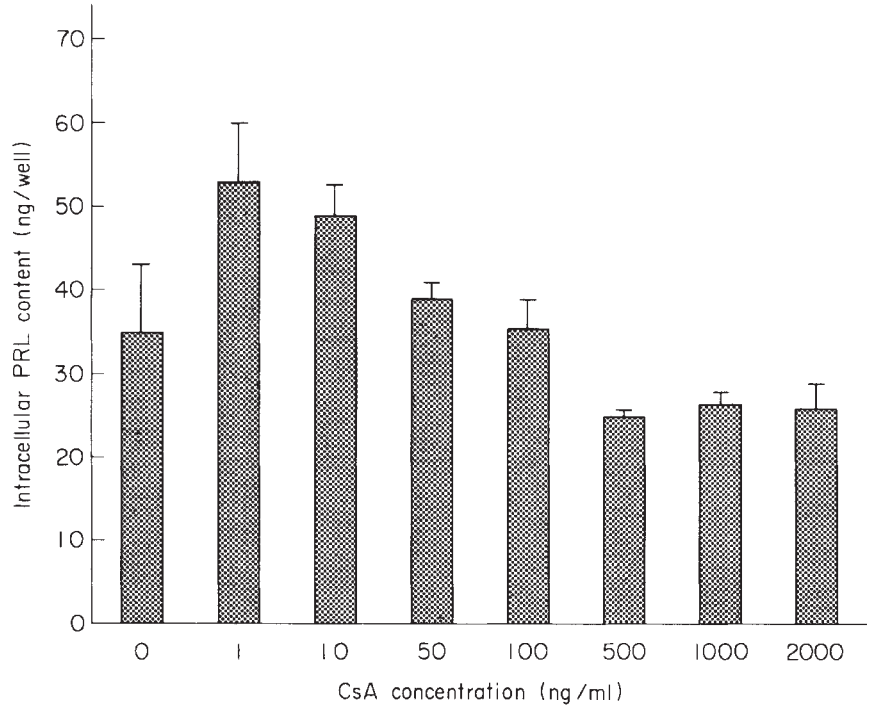


Fig. 2. The effect of CsA on the intracellular PRL content of $GH_3$ cells. After the incubation of confluent $GH_3$ cells with various concentrations of CsA for 24 hr, the intracellular PRL contents were measured by RIA, as described in Materials and Methods. The values represent the mean $\pm$ S.D. ($n=3$).

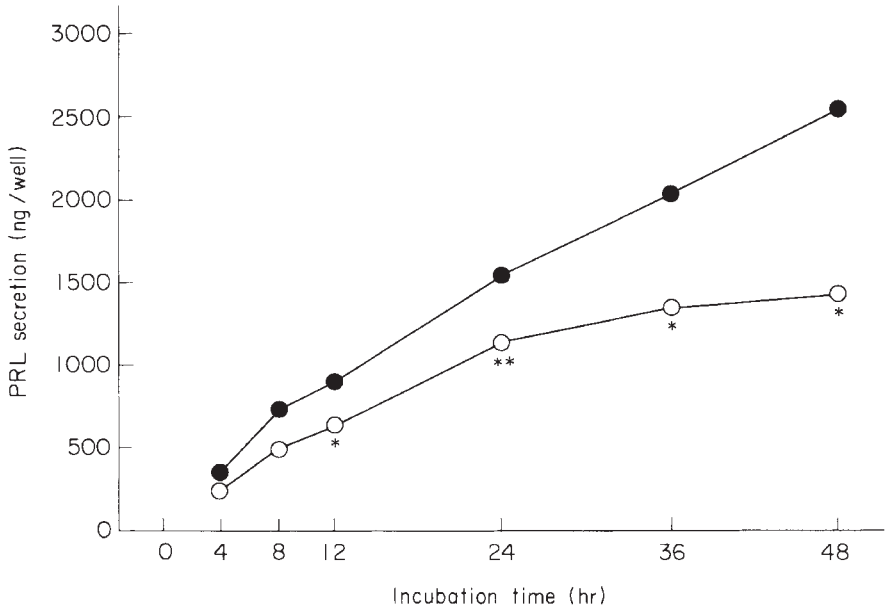


Fig. 3. Time course of the effect of CsA on PRL secretion from $GH_3$ cells. From time 0, the cells were maintained in the medium with (○) or without (●) 100 ng/ml CsA. The PRL content of the media at 4, 8, 12, 24, 36 and 48 hr were measured by RIA. Each point represents the mean of triplicate samples. $^{*}p<0.05$ and $^{**}p<0.01$ compared with control.

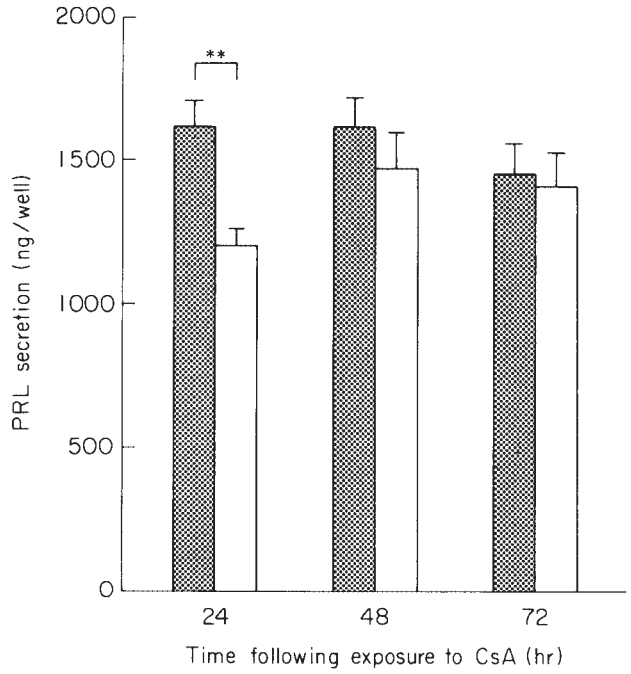


Fig. 4. Reversibility of the effect of CsA on PRL secretion from $GH_3$ cells. After incubation of confluent $GH_3$ cells in medium with (open column) or without (hatched column) 100 ng/ml CsA for 24 hr, the medium was removed, and the cells were washed twice with phosphate-buffered saline. The cells were then maintained with the fresh medium without CsA; the medium was exchanged every 24 hr for 72 hr. The PRL contents of the media were assayed every 24 hr by RIA. The values represent the mean ± S.D. ($n=3$). $^{**}p<0.01$ compared with control.

($n=3$). Although there was an apparent trend suggesting decreased viability with higher CsA concentrations, the difference between each group was not significant.

In addition, we evaluated the influence of CsA on total intracellular protein content. Various concentrations (1–2,000 ng/ml) of CsA had no effect on the total intracellular protein content (data not shown). These findings indicated that the doses of CsA which were used in this study were not cytotoxic to $GH_3$ cells.

*Northern blot analysis for PRL gene expression*

RNA strands of two distinct lengths were present: An RNA species co-migrating with 18S RNA is a possible precursor to the PRL mRNA. Northern blot analysis revealed a decrease in the PRL mRNA level in the cells treated with CsA (Fig. 5).

## Discussion

In previous in vivo studies (Cardon et al. 1984; Davenport and Hodson 1992; Nagai et al. 1992), the effect of CsA on PRL secretion and synthesis has been controversial. In addition, in vivo studies have two limitations for evaluating

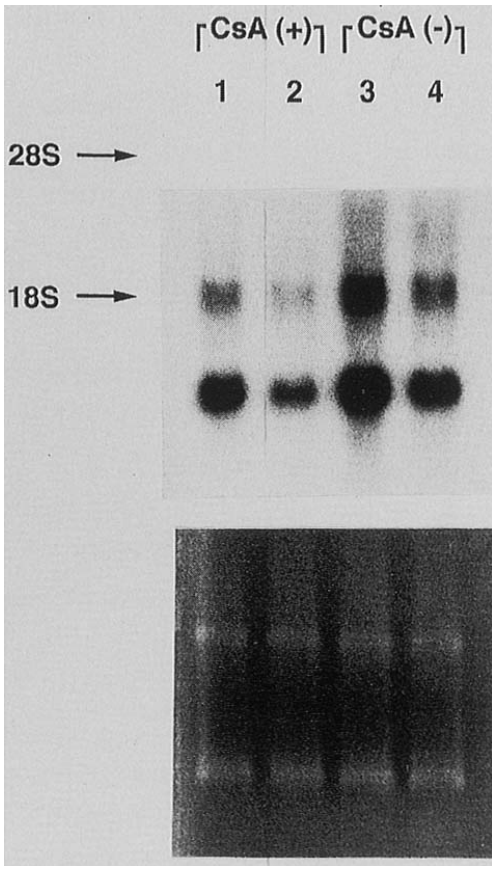


Fig. 5. Effects of CsA on the PRL mRNA content of $GH_3$ cells. After incubation with or without 100 ng/ml CsA for 24 hr, cells were harvested, and the total cellular RNA was isolated by guanidinium thiocyanate-phenol-chloroform extraction as described in Materials and Methods. Both 10 (lanes 2 and 4) and 20 $\mu$g (lanes 1 and 3) of total RNA were subjected to 1% formaldehyde-agarose gel electrophoresis and transferred to a piece of nitrocellulose filter for hybridization with oligonucleotide cDNA probe to rat PRL mRNA. Arrows indicate the relative migration of 28S and 18S RNAs run on the same gel. An RNA species co-migrating with 18S RNA is a possible precursor of PRL mRNA. Equal quantities of RNA UV trans-illumination demonstrated were applied to ethidium bromide stained agarose gel (lower). CsA (+), with 100 ng/ml CsA; CsA (−), with buffer only.

direct effect of CsA on PRL secretion. One is the possibility that CsA may act primarily on the hypothalamus, thus the effect of CsA on the pituitary gland may be secondary to a change in hypothalamic stimuli. Another possibility is that renal dysfunction induced by CsA may influence PRL secretion from the pituitary. Dysfunction of the hypothalamic-hypophyseal regulation of PRL secretion has been demonstrated in patients with chronic renal failure (Ramirez et al. 1977).

In this study, we clarified the direct effect of CsA on PRL secretion in cultured $GH_3$ cells. Our results demonstrated that CsA inhibited both PRL secretion in a dose-dependent manner and the expression of PRL mRNA in $GH_3$ cells. In contrast, CsA did not affect intracellular PRL content. It has been known that $GH_3$ cells also secrete growth hormone (GH). It is interesting whether or not CsA influences GH synthesis and secretion. However, we cannot

discuss about this question here because GH contents of the media were not measured.

Thus, the inhibitory effect of CsA on PRL secretion or synthesis is unlikely to be due to CsA cytotoxicity. Cell viability indicated by trypan blue staining was not influenced by the presence of CsA. Similarly, CsA had no effect on the total intracellular protein. Moreover, after removal of CsA from the medium, $GH_3$ cells fully recovered normal secretory activity over the next 48 hr. In addition, optimum trough serum CsA levels measured by RIA appear to be 50 to 200 ng/ml for chronic CsA administration. Therefore, the CsA concentrations used in the present study seemed to be appropriate for evaluating the direct effect of CsA on the pituitary gland.

Thus, it is reasonable to conclude that CsA decreases PRL secretion or biosynthesis in $GH_3$ cells by inhibiting the expression of PRL mRNA. However, despite the reduction of PRL mRNA, no significant changes were observed in the concentration of intracellular PRL. This result is likely due to the rapid release of newly synthesized PRL, which is characteristic of $GH_3$ cells, as previously reported (Tashjian et al. 1970).

The precise mechanism of PRL gene transcription inhibition is unclear. Recently, Wera et al. (1995) reported that transcription of the human PRL gene is inhibited by the immunosuppressants FK506, CsA and rapamycin in a pharmacological concentration range, which is in good agreement with our present results. They demonstrated the possibility that FK506- and CsA-induced inhibition of PRL gene expression in the pituitary might be mediated by calcineurin inhibition in analogy to the inhibition of IL-2 gene expression in T-lymphocytes. The hypothesis concerning the mechanism by which CsA blocks IL-2 gene expression in T cells is following: CsA enters T-lymphocytes where it initially binds an intracellular receptor termed cyclophilin (CyP) to form the CsA-CyP complex (Handschumacher et al. 1984). This CsA-CyP complex binds to, and inhibits, the $Ca^{2+}$- and calmodulin-dependent protein phosphatase termed calcineurin (Liu et al. 1991; McKeon 1991). The ability of calcineurin to dephosphorylate a cytoplasmic subunit of NF-AT (NF-ATc), a transcriptional factor implicated in the activation of IL-2 gene during T cell activation, may be blocked by the CsA-CyP complex. Subsequently, the nuclear translocation of NF-ATc may be blocked, followed by inhibition of the transcriptional activity of NF-AT (Emmel et al. 1989; DeFranco 1991). Similarly, Wera et al. (1995) suggested that the inhibitory effect of CsA on PRL gene expression may be mediated via the proximal promoter containing two *cis*-acting elements: binding sites for the transcription factor Pit-1, which is controlled through phosphorylation/ dephosphorylation, and sequence A (-115 to -85).

There is considerable evidence from in vivo studies that PRL is involved in immunomodulation. Nagy et al. (1983) have shown that lowering serum PRL levels by hypophysectomy results in a decrease in the immune response. In

addition, Russell et al. (1985) have reported the presence of PRL receptors on human T and B lymphocytes. Therefore, PRL has been regarded as a growth factor for lymphocytes or as a stimulatory factor for the immune reaction. Previous clinical studies also have demonstrated that PRL possesses immunomodulatory activity. Larson et al. (1985) have reported that heart-transplant patients with low serum PRL concentrations undergoing immunosuppressive therapy with CsA had a lower incidence of cardiac allograft rejection than patients with normal PRL concentrations. Similarly, Palestine et al. (1988) have demonstrated that lowering the serum PRL concentration with bromocriptine might be a useful adjunct to CsA therapy for autoimmune uveitis. In this study, we demonstrated an inhibitory effect of CsA on PRL synthesis. Thus, our data suggest that CsA's immunosuppressive action on helper T cells may be due to inhibiting PRL secretion in addition to its previously demonstrated effect on IL-2 production.

In conclusion, our data suggest that CsA inhibits PRL secretion by reducing the rate of biosynthesis, and that its site of action may be on PRL gene expression. However, the precise mechanism of the inhibition of PRL gene transcription remains to be elucidated. This point deserves further clarification in the future.

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