

Inhibitory Effects of Somatostatin on Rat Hepatocyte Proliferation Are Mediated by Cyclic AMP^{1,2}

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Somatostatin (SS-14) is known as an antigrowth factor for a variety of cell types, including gastrointestinal mucosa, exocrine pancreas, lymphocytes, and some tumors. We have recently identified and biochemically characterized SS-14-binding protein on rat liver plasma membranes (S. E. Raper, P. C. Kothary, and J. DelValle, *Gastroenterology* 96: A408, 1989; P. C. Kothary *et al.*, *Digestion* 46(Suppl 1): 58, 1990). We hypothesized that SS-14 may affect liver growth as well and investigated cellular mechanisms of this phenomenon focusing on the second messenger cAMP. Freshly isolated rat hepatocytes were plated on tissue culture dishes coated with Matrigel (laminin, heparan sulfate, and type IV collagen). The medium was not supplemented with serum or hormones. Either dibutyryl-cAMP (1 mM) or isobutylmethylxanthine (IBMX, 0.1 mM) was added in the presence or absence of SS-14 (10 nM). DNA synthesis was estimated by the rate of [³H]thymidine incorporation into DNA and by the labeling index (an autoradiographic measurement of the number of labeled nuclei). SS-14 significantly inhibited both [³H]thymidine incorporation and labeling index of rat hepatocytes stimulated by dibutyryl-cAMP or IBMX. SS-14 also inhibited intracellular cAMP accumulation stimulated by IBMX. We conclude that SS-14 exerts at least part of its antiproliferative effects via the adenylate cyclase system. Further study using other signal transduction systems may yield more information about mechanisms of hepatocyte growth. © 1991 Academic Press, Inc.

INTRODUCTION

Somatostatin (SS-14) is a tetradecapeptide first isolated from the hypothalamus on the basis of its ability to inhibit growth hormone secretion by rat anterior pituitary cells in culture [1]. Subsequently SS-14 has been documented to exert antigrowth effects on a variety of

cell types such as gastrointestinal mucosa [2], exocrine pancreas [3], lymphocytes [4], and some tumors [5, 6]. Its long-acting analogue (SMS 201-995) has been tried in the treatment of gut endocrine tumors with some response [7]. We have recently identified and biochemically characterized an SS-14 binding protein on rat liver plasma membranes [8, 9]. The presence of SS-14 binding protein in the liver has also been confirmed by studies demonstrating hepatic uptake of intraportally injected ¹²⁵I-[Tyr¹¹]-SS-14 in a manner partially blocked by excess unlabeled SS-14 [10]. These results led us to hypothesize that SS-14 may affect liver growth as well. There have been several reports documenting the inhibitory effects of SS-14 on liver growth [11, 12]. However, little is known about the cellular mechanisms of this antiproliferative effect.

In previous studies using other cell types, SS-14 has been shown to inhibit various cellular events by attenuating the production of cellular cyclic AMP (cAMP), an effect that seems to be mediated via pertussis toxin-sensitive inhibitory guanine nucleotide-binding proteins [13, 14]. A number of physiological findings, using cAMP analogues such as dibutyryl-cAMP (db-cAMP), suggests that attenuation of cAMP production is not sufficient to serve as the only signal for eliciting this kind of inhibition [15, 16]. Using primary cultures of adult rat hepatocytes, we investigated the cellular mechanisms of the antiproliferative effect of SS-14 focusing on the adenylate cyclase system.

MATERIALS AND METHODS

Materials

Synthetic somatostatin-14 was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Dibutyryl-cyclic AMP, 3-isobutyl-1-methyl-xanthine (IBMX), and collagenase (type I) were from Sigma Chemical Co. (St. Louis, MO). Matrigel and Dispase were obtained from Collaborative Research, Inc. (Bedford, MA).

Hepatocyte Isolation

Sprague-Dawley male rat (250–275 g) hepatocytes were isolated using a two-step collagenase perfusion

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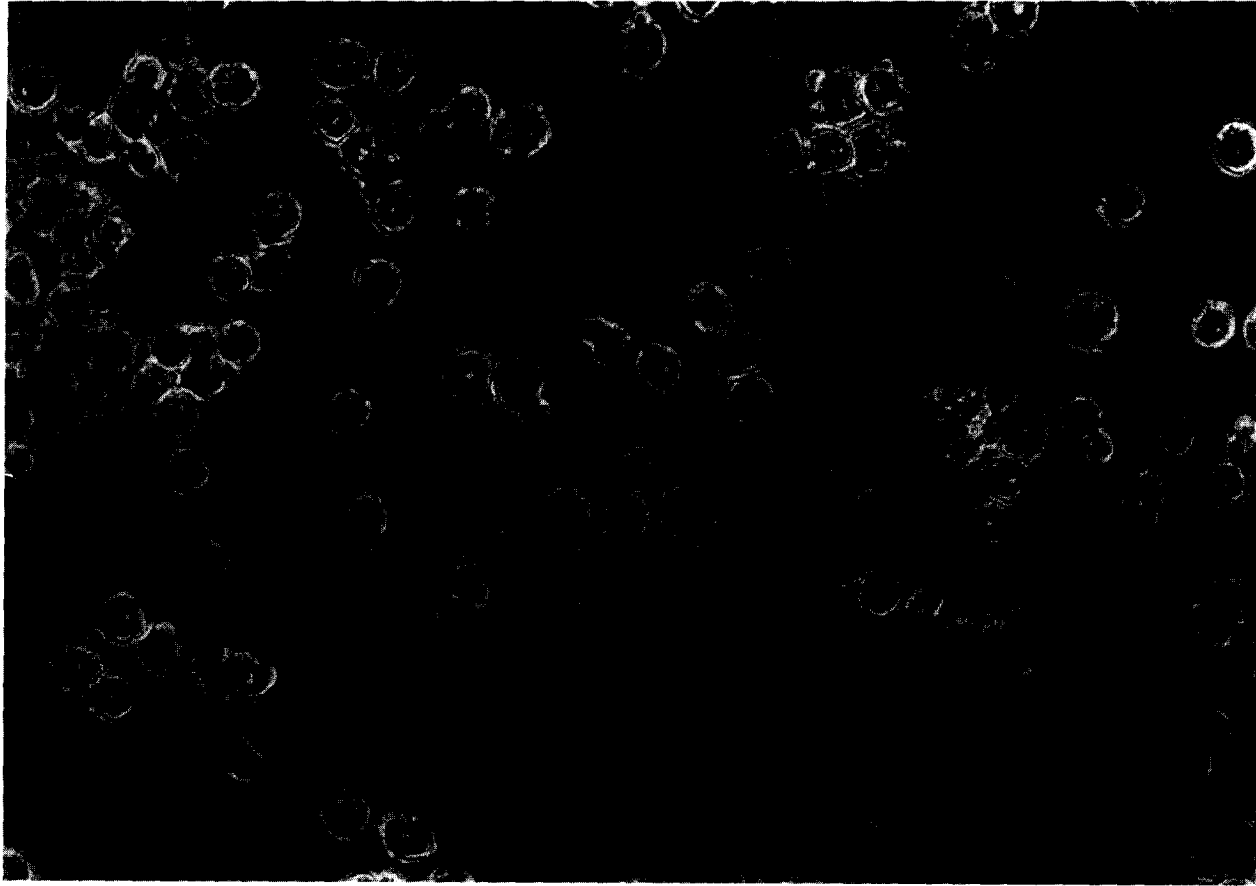


FIG. 1. Phase-contrast microscopy of rat hepatocytes cultured for 48 hr on Matrigel in presence of IBMX (0.1 mM, 24 hr) ($\times 200$).

technique [17, 18]. Briefly, rat liver was perfused for 10 min with Mg^{2+}/Ca^{2+} free Hank's buffer at $37^{\circ}C$ followed by 8–10 min with Eagle's minimum essential medium (MEM) with 0.05% collagenase (type I). The liver was excised and placed in petri dish containing fresh MEM with collagenase. The liver capsule was stripped and cells were released by gentle manipulation. The resulting crude preparation was filtered through 250 and 100 μm nylon mesh and collagenase was inactivated with 0.5 ml of fetal calf serum (FCS). Centrifugal separation was employed to minimize contamination by nonparenchymal cells. The cell viability was tested by trypan blue exclusion ($>90\%$). The hepatocytes were plated at a cell density of 10^5 per 16-mm tissue culture dish coated with 250 μl (1:3 v/v) of Matrigel (laminin, heparan sulfate, type IV collagen) [19]. When cultured on Matrigel, hepatocytes attached rapidly but exhibited minimal spreading (Fig. 1) [19], which was different from the flattened appearance of hepatocytes cultured on plastic. To check the purity of the isolated hepatocytes and to exclude a possibility of toxicity by reagents, total protein secreted by hepatocytes into the media was measured after the method of Bradford, with a Bio-Rad protein assay kit. Throughout the 48-hr experiment, there was no significant difference in protein secretion in presence

of dibutyryl-cAMP (1 mM), IBMX (0.1 mM), SS-14 (10 nM), and known hepatotrophic factors, such as insulin (100 ng/ml) or epidermal growth factor (EGF, 100 ng/

TABLE 1
Protein Concentration of Media

Reagents	Protein ($\mu g/ml$)
Control	66.7 ± 1.5
db-cAMP ^a (1 mM)	72.4 ± 10.4
+ SS-14 (10 nM)	69.6 ± 1.6
IBMX (0.1 mM)	66.3 ± 2.2
+ SS-14	70.0 ± 5.5
Insulin (100 ng/ml)	64.5 ± 6.2
+ SS-14	69.6 ± 3.1
EGF (100 ng/ml) ^b	72.0 ± 10.6
+ SS-14 ^b	66.7 ± 1.8

Note. Freshly isolated hepatocytes were plated as described under Materials and Methods. After a 24-hr attachment period, media were changed and the appropriate reagents were added. The media were collected 24 hr later, and total protein in the media was measured after the method of Bradford (BioRad, CA). Data are the mean \pm SEM from six dishes in two different experiments.

^a Dibutyryl-cAMP.

^b Insulin (100 ng/ml) was supplemented.

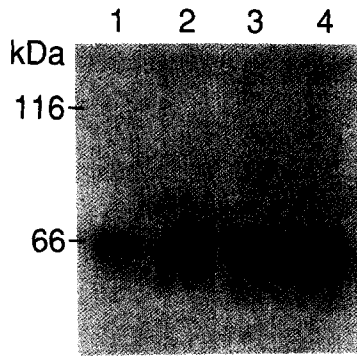


FIG. 2. Immunoblot analysis of secreted albumin into the media by rat hepatocytes. The media (25 μ l) from hepatocyte culture dishes treated with appropriate reagents were subjected to electrophoresis (SDS-PAGE) and immunoblot analysis using anti-rat albumin antibodies (Organon Teknica Corp.) [20, 21]. Lane 1, dibutyl-cAMP (1 mM); lane 2, dibutyl-cAMP + SS-14 (10 nM); lane 3, IBMX (0.1 mM); lane 4, IBMX + SS-14.

ml) (Table 1). The presence of secreted albumin in the proteins determined above was also demonstrated by an immunoblot analysis using anti-rat albumin antibodies (Organon Teknica Corp., PA) [20] (Fig. 2). Further, [35 S]cysteine incorporation into newly secreted albumin into the media was not significantly different among control, insulin (100 ng/ml), SS-14 (10 nM), and insulin + SS-14 treated hepatocytes [21], thus confirming viability of hepatocytes treated with SS-14 and/or with other reagents.

Estimation of DNA Synthesis

DNA synthesis was estimated by the rate of [3 H]thymidine incorporation into DNA [22] and by autoradiographic measurement of the number of labeled nuclei.

After a 24-hr attachment period, media were changed and the appropriate reagents were added. [3 H]thymidine (~ 2 μ Ci/dish) was added 6 hr after change of media. Using Dispase (a neutral protease produced by *Bacillus polymyxa*) [23] for 2 hr at 37°C, cells were harvested 24 hr after medium change. Half of the harvested cells were processed for counting, and the other half were used to determine DNA content. The former half was filtered onto GF/C Whatman filters and rinsed with 1 ml of ice-cold 15% trichloroacetic acid (TCA, $\times 2$) and with 70% ethanol ($\times 1$). Filters were dried and placed in 10 ml of scintillation cocktail (EcoLite) and were counted in a scintillation counter (Beckman LS 6000LL). A modified diphenylamine reaction for desoxypentose was performed to measure DNA content [24]. [3 H]thymidine incorporation was expressed as cpm/ μ g DNA.

The labeling index, i.e., the percentage of total nuclei labeled with [3 H]thymidine, was measured as follows. After an 18-hr exposure to [3 H]thymidine (2 μ Ci/dish), hepatocytes were washed twice with cold phosphate-buffered saline and fixed in 4% paraformaldehyde for 10 min, coated with Kodak NTB-3 emulsion, and exposed

for 10 days before development in Kodak Dektol. The percentage of morphologically defined hepatocytes with labeled nuclei was determined by phase contrast microscopy.

Cyclic AMP Assay

Freshly isolated hepatocytes (10^5 /ml) were incubated with the appropriate reagents for 5 min. After the incubation period, the test tubes were placed in an ice-water bath for 10 min, then vortexed gently, and centrifuged at 1800g for 10 min at 4°C. The pellet was deproteinized with 15% TCA, ether extracted, and lyophilized. Cyclic AMP levels were measured by competitive protein binding assay using an Amersham kit.

Statistical Analysis

All values shown represent the mean \pm SEM. Wilcoxon signed rank test was used for comparing group means. The level of significance was established at $P < 0.05$.

RESULTS

Effect of Dibutyl-cAMP on [3 H]Thymidine Incorporation

As shown in Fig. 3, there was dose-dependent stimulation of [3 H]thymidine incorporation by dibutyl-cAMP (10^{-7} to 10^{-3} M). The 1 mM dose was most effective and hence used for the remaining experiments.

Effect of SS-14 on Dibutyl-cAMP-Stimulated DNA Synthesis

Addition of dibutyl-cAMP (1 mM) resulted in a 252% increase in [3 H]thymidine incorporation compared to controls. SS-14 (10 nM) significantly inhibited this stimulation (Fig. 4). Of the tested doses (0.1 to 10 nM), 10 nM of SS-14 was most effective. We thus used

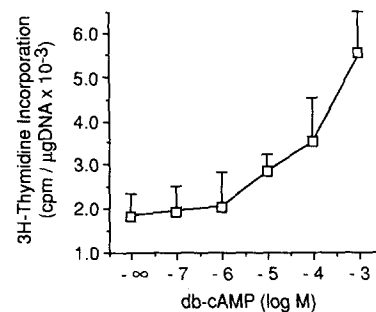


FIG. 3. Dose-dependent stimulation of [3 H]thymidine incorporation by dibutyl-cAMP (db-cAMP). Freshly isolated hepatocytes were plated as described under Materials and Methods. After a 24-hr attachment period, media were changed and dibutyl-cAMP (10^{-7} to 10^{-3} M) was added. [3 H]thymidine incorporation into DNA was measured after an 18-hr exposure to [3 H]thymidine (~ 2 μ Ci/dish). Points, means of six to eight determinations; bars, SEM.

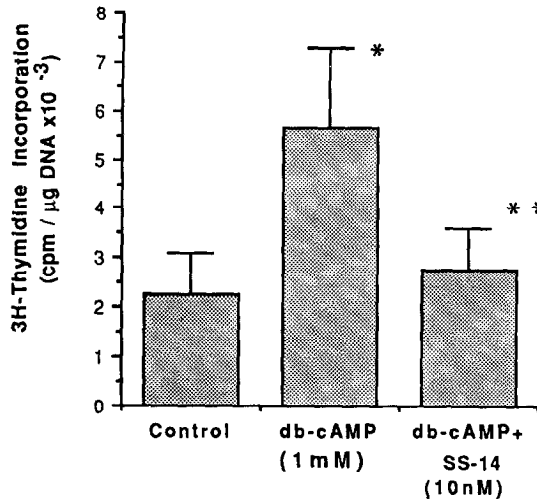


FIG. 4. Effect of SS-14 (10 nM) on dibutyryl-cAMP (1 mM)-stimulated [³H]thymidine incorporation. Methods for determining [³H]thymidine incorporation were as for Fig. 2. Columns, means from 11–12 dishes from three different experiments; bars, SEM.

P* < 0.05 vs control, *P* < 0.05 vs dibutyryl-cAMP-stimulated [³H]thymidine incorporation.

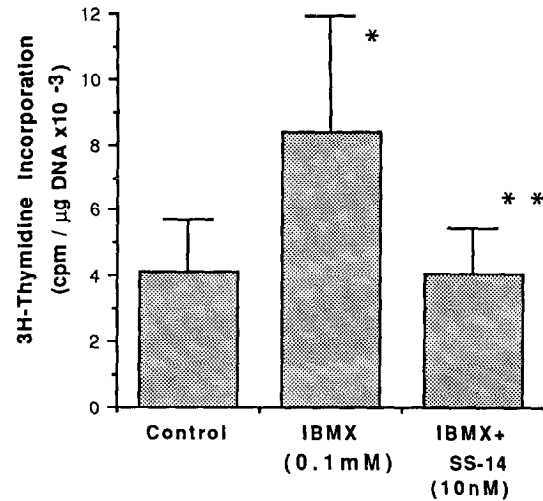


FIG. 5. Effect of SS-14 (10 nM) on IBMX (0.1 mM)-stimulated [³H]thymidine incorporation. Columns, means from 11–12 dishes from three different experiments; bars, SEM.

P* < 0.05 vs control, *P* < 0.05 vs IBMX-stimulated [³H]thymidine incorporation.

this dose of SS-14 in this study. Dibutyryl-cAMP also increased labeling index of hepatocytes, which was significantly inhibited by SS-14 (Table 2).

Effects of SS-14 on IBMX-Stimulated DNA Synthesis and Intracellular cAMP

Preliminary experiments showed that 0.1 mM IBMX was most effective in stimulation of [³H]thymidine incorporation. IBMX (0.1 mM)-stimulated [³H]thymidine incorporation and labeling index were inhibited by 10 nM SS-14 (Table 2, Fig. 5). To investigate the mechanism of this inhibitory effects, we measured the change of intracellular cAMP of rat hepatocytes. Intracellular cAMP

was significantly increased by IBMX (0.1 mM) and this response was inhibited by 10 nM SS-14 (Fig. 6).

DISCUSSION

We have shown that dibutyryl-cAMP (a nonhydrolyzable analog of cAMP) and IBMX (an inhibitor of cAMP phosphodiesterase), both of which may increase intracellular cAMP, stimulate hepatocyte DNA synthesis.

TABLE 2

Effect of SS-14 on Dibutyryl-cAMP (db-cAMP)- and IBMX-stimulated Labeling Index

Reagents	Labeling index (%)
Control	12.3 ± 2.4
db-cAMP (1 mM)	35.4 ± 5.6*
+ SS-14 (10 nM)	9.1 ± 4.4**
IBMX (0.1 mM)	37.5 ± 5.6*
+ SS-14	11.3 ± 4.4**

Note. The labeling index, i.e., the percentage of nuclei with [³H]thymidine, was measured as described under Materials and Methods. Data are the mean ± SEM from 7–10 determinations from two different experiments.

* *P* < 0.01 vs control.

** *P* < 0.01 vs db-cAMP- or IBMX-stimulated labeling index.

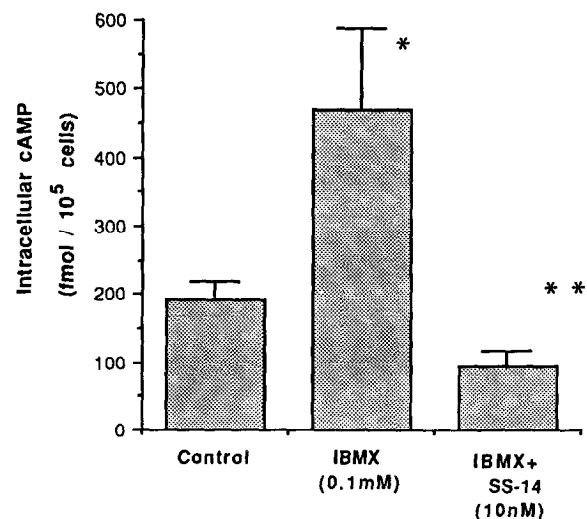


FIG. 6. Effect of SS-14 (10 nM) on IBMX (0.1 mM)-stimulated intracellular cAMP accumulation. Conditions for isolation and cAMP assay were as described under Materials and Methods. Columns, means from six tubes from two different experiments; bars, SEM.

P* < 0.05 vs control; *P* < 0.05 vs IBMX-stimulated cAMP accumulation.

Our system is different from previous studies [25–27] because we did not add any hormones or serum and we coated the culture plates with Matrigel. Using only cAMP-enhancing agents, we were able to exclude the effect of hormones or serum (including unknown factors), which may alter the adenylate cyclase activity. Thus our system was free from any endocrine stimulation. And hepatocytes were still functioning in our system as evidenced by the presence of secreted albumin. Matrigel is the first complete multicomponent preparation of natural solubilized tissue basement membrane. This laminin-rich gel is known to support differentiated functions of cultured hepatocytes for at least 3 weeks [19].

Cyclic AMP is known as one of the intracellular signals which initiate hepatocyte proliferation after partial hepatectomy [28]. Several lines of evidence obtained in studies on cultured hepatocytes [25–27] indicate that cAMP acts in the liver as a growth-promoting agent. Although there are no well-documented experiments showing physiological doses of cAMP, *in vitro* studies using dibutyryl-cAMP have documented that concentrations between 0.1 and 1 mM are effective for stimulation [25, 29–31]. Since our studies showed that dibutyryl-cAMP was most effective at 1 mM, we used this concentration to stimulate hepatocytes.

SS-14 (10 nM) significantly inhibited DNA synthesis of rat hepatocytes stimulated by both dibutyryl-cAMP and IBMX. IBMX-stimulated intracellular cAMP accumulation was inhibited by SS-14, suggesting, in part, dependence on adenylate cyclase for antiproliferative effect of SS-14.

By using cAMP analogues, we could bypass signal transduction from receptor to the production of cAMP. SS-14 is known to inhibit dibutyryl-cAMP-stimulated gastric parietal cell activity (aminopyrine uptake) [29]. Koch reported that SS-14 inhibited 8-Br-cAMP (another cAMP analogue)-stimulated prolactin release from pituitary cells [13]. In our study, using rat hepatocytes, SS-14 significantly inhibited dibutyryl-cAMP-stimulated [³H]thymidine incorporation. The data suggest that inhibitory actions of SS-14 are both proximal and distal to activation of adenylate cyclase. Similar observations have recently been reported in the growth study using the FRTL5 line of rat thyroid follicular cells [32].

In conclusion, SS-14 may exert at least part of its antiproliferative effect via the adenylate cyclase system. However, we must investigate the possibility that the antiproliferative effect of SS-14 may be mediated via multiple pathways because SS-14 has recently been shown to inhibit DNA synthesis of thyroid follicular cells stimulated by insulin-like growth factor I, which is not dependent on cAMP [32]. Further study on other signal transduction systems may yield more information about mechanisms of hepatocyte growth.

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