

## Vasopressin Stimulates DNA Synthesis in Cultured Rat Hepatocytes<sup>1,2</sup>

FAIZULLAH Y. BHORA, M.D., PIYUSH C. KOTHARY, M.S., HIROAKI IMANISHI, M.D., FREDERIC E. ECKHAUSER, M.D., AND STEVEN E. RAPER, M.D.

Department of Surgery, University of Michigan Medical School, Ann Arbor, Michigan 48109

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Liver regeneration following partial hepatectomy is significantly impaired in rats with hereditary vasopressin (AVP) deficiency. This suggested that AVP might have a direct effect on cultured rat hepatocytes. Hepatocytes from male Sprague-Dawley rats were isolated using a two-step collagenase perfusion technique and plated at a density of  $10^5/16$ -mm Primaria plate. After a suitable attachment period, hepatocytes were incubated with minimal essential media, AVP, AVP plus a specific AVP antagonist, or oxytocin. Hepatocyte proliferation was measured by [<sup>3</sup>H]thymidine incorporation ([<sup>3</sup>H]Thy) into hepatocyte DNA. AVP (10 nM) increased [<sup>3</sup>H]Thy significantly (and this effect was blocked by an AVP-specific antagonist (50 nM). Oxytocin had no effect on hepatocyte DNA synthesis. To further investigate the influence of AVP on hepatocyte proliferation, the effect of AVP on transforming growth factor- $\alpha$  (TGF- $\alpha$ )-stimulated hepatocyte proliferation was also studied. This combination was chosen based on the ability of AVP to inhibit the biologic effects of EGF (a TGF- $\alpha$  analog). There was significant attenuation of TGF- $\alpha$  (50 nM)-stimulated [<sup>3</sup>H]Thy in the presence of AVP (10 nM). In summary: (1) AVP stimulates proliferation of cultured rat hepatocytes. (2) The effect of AVP can be significantly abolished by a specific AVP antagonist. (3) The proliferative response of AVP is specific. (4) AVP significantly attenuates TGF- $\alpha$ -stimulated hepatocyte hepatic DNA synthesis. Further studies should elucidate the mechanisms for the effects of AVP on hepatic proliferation alone or in combination with other factors. © 1994 Academic Press, Inc.

### INTRODUCTION

Arginine<sup>8</sup> vasopressin (AVP) is a nonapeptide released by the posterior pituitary into the peripheral cir-

ulation. It is used frequently as a means to control acute variceal hemorrhage in patients with portal hypertension and is nonspecifically associated with the volume changes associated with many major surgical procedures [1]. Although no detailed studies are available on the levels of AVP after hepatic resection in normal rats, suggestive evidence is provided by the Brattleboro rat, which is deficient in AVP and has impaired hepatic regeneration. The impaired proliferation rate can be corrected by the administration of exogenous AVP [2]. Also, AVP has been noted to potentiate growth factor-stimulated DNA synthesis in cultured rat hepatocytes [3]. The effects of AVP alone have not been studied in cultured rat hepatocytes, where the relative contribution of a direct vasopressin effect on hepatocyte proliferation may be distinguished from secondary effects due to altered blood flow.

Hepatic and hemodynamic effects of AVP on the liver are mediated by vasopressin receptors of the V<sub>1</sub> subtype [4]. The direct effect of AVP on hepatocyte proliferation was studied *in vitro* in primary cultures of rat hepatocytes. To further study the specificity of AVP-mediated rat hepatocyte proliferation, oxytocin, which differs from AVP at amino acid positions 3 and 8, was administered to hepatocytes. A review by Manning and Sawyer has detailed the fact that although oxytocin can bind to the V<sub>1a</sub> receptor, it does not produce a biologic effect [5]. The data suggest that variations in amino acid sequence make a significant difference in biologic activity. A competitive antagonist of AVP, [ $\beta$ -mercapto- $\beta$ , $\beta$ -cyclopentamethylenepropionyl<sup>1</sup>, O-Me-Tyr<sup>2</sup>, Arg<sup>8</sup>]vasopressin ( $\beta$ -ME AVP) was administered to hepatocytes alone or in combination with AVP.

Recently, AVP has been shown to inhibit receptor-mediated endocytosis of dissimilar peptides including epidermal growth factor (EGF), a potent hepatotrophic factor, and other proteins such as asialoglycoprotein [6, 7]. Transforming growth factor- $\alpha$  (TGF- $\alpha$ ) shares a 40% homology with EGF, binds to the same receptor *in vivo*, and is the physiologically relevant peptide in hepatocyte proliferation [8]. To determine the effect of AVP on TGF- $\alpha$ -stimulated hepatocyte proliferation *in vitro*, TGF- $\alpha$  alone, or in combination with AVP.

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## MATERIALS AND METHODS

### Chemicals

Collagenase (type 1), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), AVP, oxytocin, glucagon,  $\beta$ -ME AVP, TGF- $\alpha$ , and rat serum albumin were purchased from Sigma Chemicals Co. (St. Louis, MO). All reagents for polyacrylamide gel electrophoresis and immunoblot analysis were obtained from Bio-Rad Co. (Richmond, CA). [ $^3$ H]Thymidine (7 Ci/mmol) was obtained from Amersham Corp (Arlington Heights, IL).

### Animals

Male Sprague-Dawley rats (200–275 g) were purchased from the Charles River Laboratories (Wilmington, DE). Prior to hepatocyte isolation and sacrifice, animals were housed in temperature- and light-controlled rooms and received food and water *ad libitum*. Institutional guidelines for the care and use of animals were strictly adhered to.

### Hepatocyte Isolation

All hepatocytes were derived from primary cultures obtained from the liver of normal rats using a two-step collagenase perfusion technique described previously [9–12]. The cultures were all prepared fresh and used on the day of isolation. For each experimental manipulation, triplicate cultures were used, and 3 to 5 rats were used for the isolated cultures (accounting for experimental numbers of 9–15). Rats were anesthetized with isoflurane and supplemental oxygen (Forane; Anaquest, Madison, MI) [13]. The technique of hepatocyte isolation was as previously reported. Briefly, after laparotomy, the inferior cava was cut, and the portal vein was cannulated with polyethylene tubing. The liver was then perfused with  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks' buffer (GIBCO, Grand Island, NY) containing 30 mM Hepes at 37°C for about 10 min at a flow rate of 10 ml/min. The perfusate was then changed to Eagle's minimal essential medium (MEM, GIBCO) containing 0.05% collagenase (type 1) for 10–15 min or until the capsule began to separate from the underlying hepatocytes. The liver was excised and placed in a sterile petri dish with fresh collagenase-containing medium. The capsule was stripped away and the cells were gently released by manipulation. The resulting crude suspension was filtered through four layers of cheesecloth. Collagenase was inactivated by the addition of 0.5 ml of 10% fetal calf serum (GIBCO) per 10 ml of cell suspension and two subsequent low-speed centrifugations in fresh MEM at 4°C. Cells were suspended in fresh MEM, and an aliquot was counted for cell number and viability with Trypan blue. Only preps with viability in excess of 85% were accepted for use. Hepatocyte suspensions were plated at a density of  $10^5$  per 16-mm Pri-

maria tissue culture well (Becton-Dickinson Co., Lincoln Park, NJ).

To evaluate differentiated hepatocyte function, hepatocytes were kept in 5%  $\text{CO}_2$  and examined daily for degree of attachment and morphology. Aliquots of medium were analyzed for the presence of rat albumin secreted into the medium by SDS-polyacrylamide gel electrophoresis. The level of albumin secretion did not vary with experimental conditions, suggesting that toxicity due to reagents was negligible (data not shown).

### Quantitation of DNA Synthesis

DNA synthesis was measured by the rate of [ $^3$ H]Thy incorporation into DNA. After a 2-hr attachment period in serum-free MEM with 10% FCS, fresh medium was added and cells were incubated overnight. Experimental reagents were added in fresh serum-free medium, and each treatment was performed in triplicate. [ $^3$ H]Thy was added 6 hr after addition of reagents (2  $\mu\text{Ci}/\text{well}$ ), and trypsin was used to release cells 18 hr after the addition of the [ $^3$ H]Thy quantitation. Half of the resulting cell suspension was used to quantitate DNA. DNA analysis was performed by a colorimetric analysis using a diphenylamine reaction for deoxypentose [14]. Cells used for [ $^3$ H]Thy quantitation were layered onto glass microfiber filters (Whatman International, Maidstone, England) and rinsed with 15% ice-cold trichloroacetic acid twice, followed by a wash with ice-cold 70% ethanol. Filters were dried, placed in 10 ml scintillation cocktail (Ecolite; ICN Biochemicals Inc., Irvine, CA), and counted in a Beckman LS 6000 LL scintillation counter (Beckman Instruments Inc., Fullerton, CA). The same counter was used for each analysis and calibrated at frequent intervals to insure the same efficiency of counting. Results were expressed as cpm/ $\mu\text{g}$  DNA.

### Statistical Analysis

All analyses were done using the nonparametric Wilcoxon signed rank test on a Macintosh SE with StatWorks software. Significance was accepted at  $P < 0.05$  [15].

## RESULTS

Appropriate dose-response curves were performed using AVP, glucagon, a peptide with minimal hepatotropic action but significant effects on hepatic physiology, oxytocin as a peptide structurally similar to AVP, and TGF- $\alpha$  (Table 1). Such studies determined the concentrations of peptide used in subsequent experiments: AVP, 10 nM; TGF- $\alpha$ , 50 nM; oxytocin, 50 nM; and glucagon 100 nM. As expected, neither glucagon ( $P > 0.01$ ) nor oxytocin ( $P > 0.169$ ) demonstrated any significant effect on [ $^3$ H]Thy incorporation.

The effect of  $\beta$ -ME AVP on AVP-stimulated hepatocyte proliferation was assessed by culture of hepatocytes

**TABLE 1**  
**Peptide Dose-Response Curve**

Concentration (nM)	<sup>3</sup> H]Thymidine incorporation (cpm/μg DNA)			
	AVP (%)	Glucagon (%)	TGFα (%)	Oxytocin (%)
MEM	790 ± 91 (100)	1845 ± 289 (100)	1845 ± 289 (100)	
0.1	967 ± 128 (122)			
1.0	2068 ± 662 (262)	2232 ± 462 (121)	1858 ± 159 (101)	1954 ± 348 (100)
10	2677 ± 1409 (339)*	2232 ± 421 (121)	1892 ± 225 (103)	1217 ± 225 (62)
25			2246 ± 343 (122)	
50			3338 ± 1069 (181)*	917 ± 512 (47)
100		2594 ± 627 (141)		

\*  $P < 0.05$  by Wilcoxon's signed rank test.

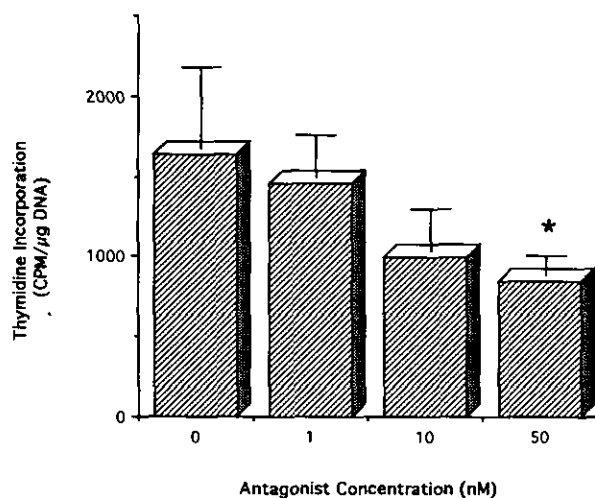
in the presence of AVP,  $\beta$ -ME AVP, or both. Figure 1 represents the dose response of hepatocytes incubated with 10 nM AVP and increasing concentrations of  $\beta$ -ME AVP. As noted, the most significant inhibitory effect is seen at 50 nM of antagonist. To further characterize the peptide specificity of the hepatocyte DNA synthetic response, a lack of significant stimulation was noted with oxytocin, an effect similar to that of medium alone, or to the combination of AVP and  $\beta$ -ME AVP (Fig. 2).

An intriguing finding, previously reported, was the fact that the effects of epidermal growth factor inhibited *in vitro* by the administration of AVP [8]. Given the sequence homology of EGF and TGF- $\alpha$  it was of interest to determine whether or not TGF- $\alpha$ -mediated hepatocyte proliferation could be inhibited by AVP *in vitro*. Figure 3 represents the effects of AVP, TGF- $\alpha$ , or both. While significantly stimulating proliferation separately,

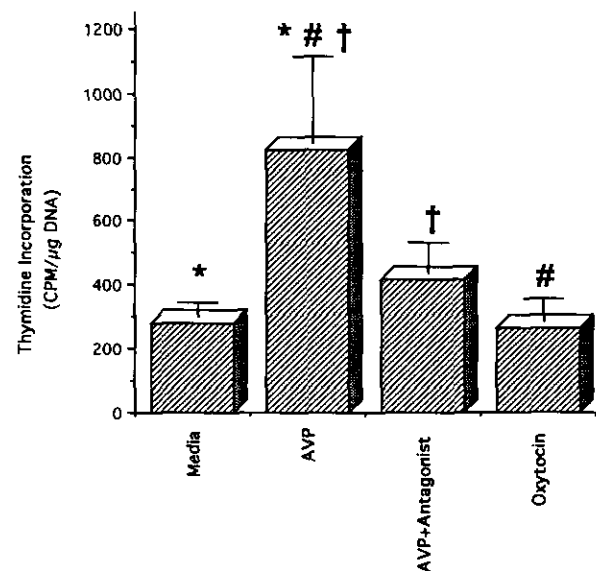
a decreased proliferative effect was noted when combined. Conversely, glucagon had no effect on AVP-stimulated <sup>3</sup>H]Thy incorporation into DNA (Fig. 4).

## DISCUSSION

These experiments have documented the hepatotropic properties of AVP in minimal defined medium, in the absence of other known hepatotropic factors. The measurement of <sup>3</sup>H]thymidine incorporation into DNA is a simple quantitative method for evaluation of cellular proliferation [16]. Although binding studies were not done in the context of these experiments, the data is compatible with a hypothesis of receptor-mediated action. AVP is known to exert biologic effects via two dis-



**FIG. 1.** Dose response of 10 nM AVP-stimulated thymidine incorporation in the presence of [ $\beta$ -mercapto  $\beta$ , $\beta$ -cyclopentamethylene-propionyl<sup>1</sup>, O-Me-Tyr<sup>2</sup>, Arg<sup>8</sup>]vasopressin ( $\beta$ -ME-AVP). The addition of  $\beta$ -ME AVP inhibited AVP-stimulated proliferation in a dose-dependent manner ( $n = 9-15$ ; mean  $\pm$  SEM; \* $P < 0.05$ ).



**FIG. 2.** Effect of 10 nM AVP on thymidine incorporation in the presence of  $\beta$ -ME AVP. The addition of  $\beta$ -ME AVP inhibited AVP-stimulated proliferation, while oxytocin, a vasopressin analogue that has been shown to bind the  $V_1$  receptor subtype, had no effect ( $n = 9-15$ ; mean  $\pm$  SEM; \* $P < 0.01$ ).

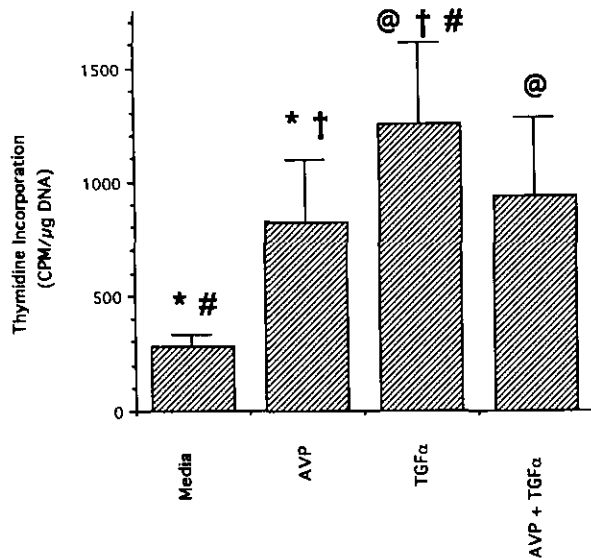


FIG. 3. Effect of AVP (10 nM) or TGF- $\alpha$  (50 nM) on [ $^3$ H]Thy incorporation. The addition of both AVP (10 nM) and TGF- $\alpha$  (50 nM) resulted in less [ $^3$ H]Thy incorporation than TGF- $\alpha$  alone ( $n = 9-15$ ; mean  $\pm$  SEM; \* $\dagger$ , $\#$   $P < 0.01$ ).

tinct receptor subtypes  $V_1$  and  $V_2$ .  $V_1$  type receptors are found in the vascular system and the liver;  $V_2$ -type receptors are located primarily in the kidney [4]. The different receptor subtypes appear to interact via different intracellular pathways.  $V_1$  receptor stimulation results in activation of phospholipase C with subsequent formation of diacylglycerol and inositol 1,4,5-trisphosphate, while  $V_2$  receptors appear to activate adenyl cyclase [17]. The former releases intracellular calcium, while the latter activates cyclic AMP dependent protein kinase [18-25]. DNA synthesis may require a priming period related to cyclic AMP dependent protein kinase activation in primary hepatocyte cultures [26].

The amino acid sequence appears to be important in the hepatic proliferative effects of AVP. If the phenylalanine at position three is substituted for isoleucine, and the arginine at position 8 is substituted for leucine, as in oxytocin, no proliferative response is noted (Fig. 2). In addition, the proliferative effect of AVP can be blocked by specific antagonists, such as  $\beta$ -ME AVP, which has a high affinity for the  $V_1$ -type receptor [27]. A 50% attenuation of the AVP proliferative response was seen in response to antagonist (Figs. 1 and 2).

Liver regeneration is a precisely regulated biologic phenomenon, influenced by myriad hepatic and extrahepatic factors. Both TGF- $\alpha$  and glucagon have been documented to be hepatotrophic agents, either alone or in combination with other factors [6, 8, 28]. AVP has been documented to decrease the affinity of the EGF receptor for EGF, without any decrease in receptor number [8]. The biologically active fragment of TGF- $\alpha$  is a 50-amino-acid peptide originally detected in the culture fluid of retrovirally transformed cell lines [29]. TGF- $\alpha$

has a 33-44% homology with EGF and binds to the same cell surface receptor as EGF. Comparative studies indicate that TGF- $\alpha$  is a more effective hepatocyte proliferative agent than EGF [30-31]. TGF- $\alpha$  is believed to be one of the physiologic effectors of hepatic regeneration, playing a role as a promoter of regeneration [32]. Although binding per se was not studied, the data presented here suggest that AVP and TGF- $\alpha$  act to inhibit, rather than synergistically enhance, mutually observed proliferative effects (Fig. 3). The data are compatible with a hypothesis that AVP may decrease the affinity of TGF- $\alpha$  for its receptor. Such data, however, does not exclude interference in intracellular second messenger signaling.

The interaction of AVP and glucagon on hepatocyte proliferation was also evaluated. Glucagon, like AVP, is known to activate PKC [20, 21]. No effect was noted when glucagon was added to AVP-stimulated hepatocytes, consistent with a shared intracellular second-messenger pathway (Fig. 4).

In summary, AVP stimulates cultured rat hepatocytes in a dose-dependent fashion. The proliferative response was abolished by a specific AVP antagonist, and dependent on structure, as noted by the lack of effect of oxytocin. With AVP and TGF- $\alpha$  together, a partial inhibitory response was noted, compatible with a hypothesis of alteration in TGF- $\alpha$  binding. The splanchnic vasoconstrictive effects of AVP have long been considered detrimental to nutritive flow to the liver. The ability of AVP to promote hepatocyte proliferation *in vitro* suggests that *in vivo* effects are complex. Future studies into the mechanisms of the AVP-mediated proliferative re-

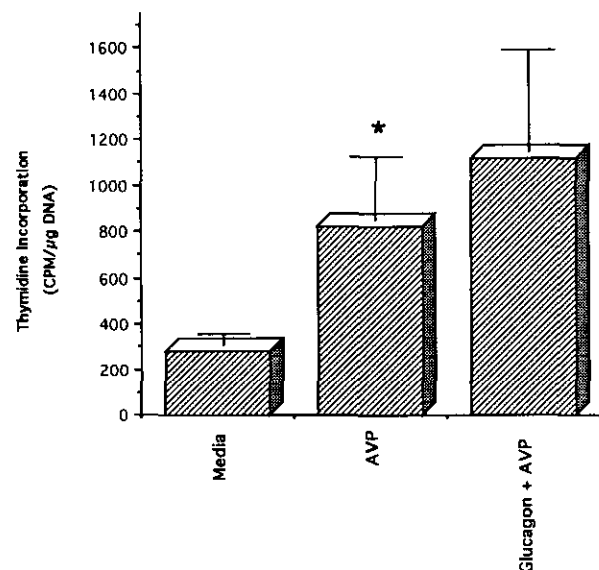


FIG. 4. Effect of AVP (10 nM) or glucagon (100 nM) on [ $^3$ H]Thy incorporation. The addition of both AVP (10 nM) and glucagon (100 nM) resulted in the same [ $^3$ H]Thy incorporation as AVP alone ( $n = 9-15$ ; mean  $\pm$  SEM; \* $P < 0.01$ ).

sponse will provide a better understanding of liver regeneration.

#### REFERENCES

1. Wait, R. B., and Kahng, K. U. Fluids and electrolytes and acid-base balance. In L. J. Greenfield, M. W. Mulholland, K. T. Oldham, and G. B. Zelenock (Eds.), *Surgery: Scientific Principles and Practice*. Philadelphia, PA: Lippincott, 1992. Pp. 224-225.
2. Russell, W. E., and Bucher, N. L. R. Vasopressin modulates liver regeneration in the Brattleboro rat. *Am. J. Physiol.* **245** (*Gastrointest. Liver Physiol.* **8**): G321, 1983.
3. Russell, W. E., and Bucher, N. L. R. Vasopressin augments growth factor-stimulated DNA synthesis in primary cultures of adult rat hepatocytes. *Endocrinology* **110**: A162, 1982.
4. Mitchell, R. H., Kirk, C. J., and Billah, M. M. Hormonal stimulation of phosphatidylinositol breakdown, with particular reference to the hepatic effects of vasopressin. *Biochem. Soc. Trans.* **7**: 861, 1979.
5. Manning, M., and Sawyer, W. H. Discovery, development, and some uses of vasopressin and oxytocin antagonists. *J. Lab. Clin. Med.* **114**: 617, 1989.
6. Mead, J. E., and Fausto, N. Transforming growth factor  $\alpha$  may be a physiological regulator of liver regeneration by means of an autocrine mechanism. *Proc. Natl. Acad. Sci. USA* **86**: 1558, 1989.
7. Gil-Falgon, S., Lamaze, C., Haccin-Bey, S., and Feger, J. Effects of vasopressin on receptor-mediated endocytosis of asialoglycoprotein by hepatocytes from normal and diabetic rats. *Exp. Cell Res.* **199**: 223, 1992.
8. Rozenfurt, E., Brown, K. D., and Pettican, P. Vasopressin inhibition of epidermal growth factor binding to cultured mouse cells. *J. Biol. Chem.* **257**: 716, 1981.
9. Raper, S. E., Kothary, P. C., and Kokudo, N. Somatostatin-14 blocks the hepatotrophic effects of insulin in the rat. *J. Surg. Res.* **50**: 386, 1991.
10. Kokudo, N., Kothary, P. C., Eckhauser, F. E., and Raper, S. E. Inhibitory effects of somatostatin on hepatocyte proliferation are mediated by cyclic AMP. *J. Surg. Res.* **51**: 113, 1991.
11. Kokudo, N., Kothary, P. C., Eckhauser, F. E., Nakamura, T., and Raper, S. E. Inhibition of DNA synthesis by somatostatin in rat hepatocytes stimulated by hepatocyte growth factor or epidermal growth factor. *Am. J. Surg.* **163**: 169, 1992.
12. Seglen, P. O. Preparation of isolated rat liver cells. *Methods Cell Biol.* **13**: 29, 1976.
13. Raper, S. E., Barker, M. E., Burwen, S. J., and Jones, A. L. Isoflurane as an experimental anesthetic. *Anat. Rec.* **218**: 116, 1987.
14. Volkin, E., and Cohn, W. E. Estimation of nucleic acids. *Methods Biochem. Anal.* **1**: 287, 1956.
15. Colton, T. Non-parametric methods. In *Statistics in Medicine*. Boston: Little, Brown, 1974.
16. Bucher, N. L. R., and Swaffield, M. N. The role of incorporation of labeled thymidine into the deoxyribonucleic acid of regenerating rat liver in relation to amount of liver excised. *Cancer Res.* **24**: 1611, 1964.
17. Sato, C., Liu, J., Uchihara, M., Izumi, N., Yauchi, T., Sakaj, Y., Asahina, Y., Fukuma, T., Takano, T., and Marumo, F. VI-receptor mediated GSH efflux by vasopressin from rat hepatocytes. *Life Sci.* **51**: 507, 1992.
18. Diaz-Guerra, M. J. M., and Bosca, L. Lack of translocation of protein kinase C from the cytosol to the membranes in vasopressin-stimulated hepatocytes. *Biochem. J.* **269**: 163, 1990.
19. Tang, E. K. Y., and Houslay, M. D. Glucagon, vasopressin and angiotensin all elicit a rapid, transient increase in hepatocyte protein kinase C activity. *Biochem. J.* **283**: 341, 1992.
20. Pittner, R. A., and Fain, N. J. Activation of membrane protein kinase C by glucagon and  $Ca^{2+}$  mobilizing hormones in cultured rat hepatocytes. *Biochem. J.* **281**: 371, 1991.
21. Wallace, M. A., Randazzo, P., Li, S., and Fain, J. N. Direct stimulation of phosphatidylinositol degradation by addition of vasopressin to purified rat liver plasma membranes. *Endocrinology* **111**: 341, 1982.
22. Kirk, C. J., Mitchell, R. H., and Hems, D. A. Phosphatidylinositol metabolism in rat hepatocytes stimulated with vasopressin. *Biochem. J.* **194**: 155, 1981.
23. Lin, S. H., and Fain, J. N. Vasopressin and epinephrine stimulation of Phosphatidylinositol breakdown in the plasma membrane of rat hepatocytes. *Life Sci.* **29**: 1905, 1981.
24. Marino, M., Mangiantini, M. T., Spagnuolo, S., Luly, P., and Leoni, S. Signal transduction during liver regeneration: role of insulin and vasopressin. *J. Cell. Physiol.* **152**: 403, 1992.
25. Cerpovicz, P. F., and Ochs, R. S. Effects of EGF on the mass of inositol 1,4,5-triphosphate and SN (1,2)-diacylglycerol in freshly isolated rat hepatocytes: Comparison with vasopressin. *Biochem. Biophys. Res. Commun.* **187**: 1055, 1992.
26. Hirata, K., and Fujiwara, K. Possible contribution of protein kinase C activation to priming for DNA synthesis induced by epidermal growth factor with insulin and its inhibition by plasma membrane in primary cultured rat hepatocytes. *Biochem. Biophys. Res. Commun.* **171**: 1093, 1990.
27. Kruszynski, M., Lammek, B., and Manning, M. [1-( $\beta$ -Mercapto- $\beta$ , $\beta$ -cyclopentamethylenepropionic acid)]L-arginine-vasopressin, two highly potent antagonists of the vasopressor response to arginine-vasopressin. *J. Med. Chem.* **23**: 364, 1980.
28. McGowan, J. A. Hepatocyte proliferation in culture. In A. Guillonzo and C. Guguen-Guillonzo (Eds.), *Research in Isolation and Cultured Hepatocytes*. London: John Libbey, 1986. Pp. 14-38.
29. De Larco, J. E., and Todaro, G. J. Growth factor from murine sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. USA* **75**: 4001, 1978.
30. Massague, J. Epidermal growth factor-like transforming growth factor, 1. Isolation, chemical characterization, and potentiation by other transforming factors from feline sarcoma virus-transformed rat cells. *J. Biol. Chem.* **258**: 13606, 1983.
31. Lee, D. C., Rose, T. M., Webb, N. R., and Todaro, G. J. Cloning and analysis of cDNA for rat transforming growth factor- $\alpha$  stimulates proto-oncogene *c-jun* expression and a mitogenic program in primary cultures of adult rat hepatocytes. *DNA* **8**: 289, 1989.
32. Fausto, N., and Mead, J. E. Regulation of liver growth: Protooncogenes and transforming growth factors. *Lab. Invest.* **60**: 4, 1989.