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Regulation of Hepatocyte Growth: Alpha-1 Adrenergic Receptor and *ras* p21 Changes in Liver Regeneration

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Catecholamines, acting via the alpha-1 adrenergic receptor, have been demonstrated to influence adult rat hepatocyte DNA synthesis in primary culture and in vivo during liver regeneration following partial hepatectomy (PHX). Earlier investigations have suggested that the alpha-1 effect on DNA synthesis is significant only during the first day following PHX. We examined receptor binding at several early and late time points after surgery, and we observed a significant loss of specific [^{3}H]-prazosin binding to cells isolated from rat livers 48 and 72 hr after PHX. In contrast, the ability of norepinephrine to stimulate inositol phosphate production in isolated cells prelabeled with [^{3}H]-myo-inositol was transiently reduced between 8 and 16 hr, when alpha-1 binding capacity was virtually unchanged. This uncoupling of phosphoinositide turnover from binding was preceded by a drop in hepatic membrane *ras* p21 content, as assayed by liquid competition radioimmunoassay. The loss of immunoreactive p21 from membranes was significant by 2 hr after PHX. These findings suggest a role for alpha-1 receptors and *ras* protein in the early events of liver regeneration.

The primary function of the hepatic alpha-1 adrenergic receptor is understood to be the regulation of liver glycogen storage through its control of enzymes of glycogenolysis and gluconeogenesis. Under conditions of primary cell culture and regeneration, however, this same receptor is apparently involved in triggering entry into S phase. What is the role of this receptor in rat hepatocyte DNA synthesis, and what determines whether its stimulation affects glucose homeostasis or growth?

Looking first at an in vitro model of stimulated DNA synthesis in primary hepatocyte cultures, we demonstrated that alpha-1 adrenergic receptors mediate an enhancement of DNA synthesis by norepinephrine (NE) (Cruise and Michalopoulos, 1985; Cruise et al., 1985, 1986). More recently, we have shown that alpha-1 receptor blockade inhibits regenerative DNA synthesis, implicating this receptor in the early triggering events after partial hepatectomy (Cruise et al., 1987). Alpha-1 adrenergic receptors are part of a family of receptors coupled through phospholipase C (PLC) to the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP2) in membranes, and the effects of alpha-1 agonists are presumably mediated by the second messengers generated from this breakdown: inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). To further investigate the role of alpha-1 adrenergic receptors in liver regeneration, we have now monitored not only the binding capacity of cells isolated from regenerating livers but also the coupling of alpha-1 adrenergic receptor occupancy to the generation of inositol phospholipid breakdown products.

Coupling of alpha-1 adrenergic and other receptors to PLC is through an uncharacterized guanine nucleotide binding protein (G protein) (Goodhardt et al., 1982). A membrane-associated G protein of unknown function that appears to be involved in the regulation of cell growth is the product of the *ras* proto-oncogene (p21). While *ras* mRNA expression during liver regeneration has been described (Thompson et al., 1986), membrane p21 protein levels have not. In this paper we also report findings from assays of hepatic membrane p21 in regeneration.

Our data show that an early fall in the p21 content of liver membranes precedes a transient uncoupling of alpha-1 adrenergic receptor binding from PIP2 breakdown and that a down-regulation of alpha-1 receptors occurs later, at 48–72 hr after partial hepatectomy.

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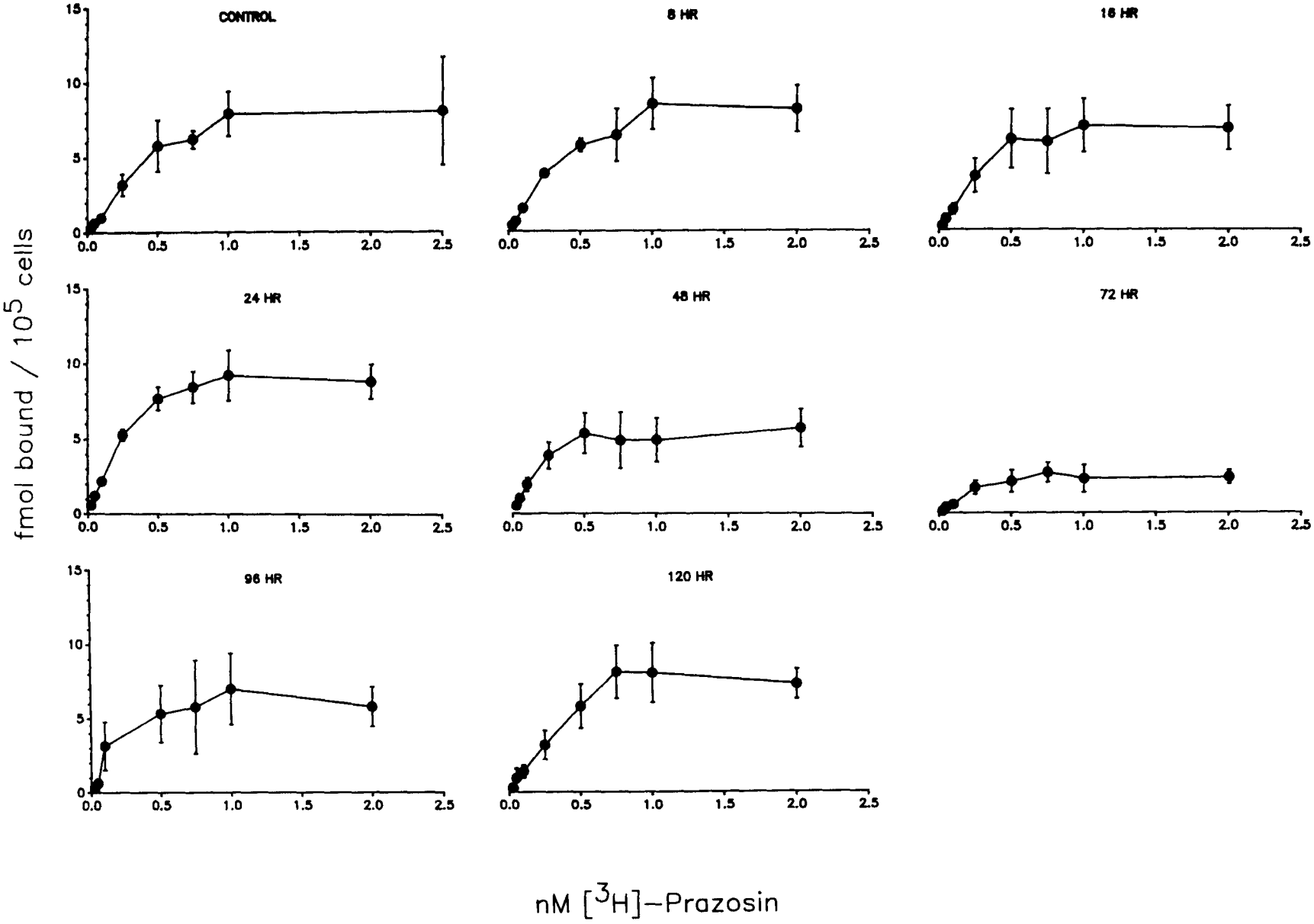
Abbreviations used: BSA, bovine serum albumin; DAG, 1,2-diacylglycerol; EGTA, ethyleneglycol-bis-(beta-aminoethyl ether) N,N,N′,N′-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IP, inositol phosphates; IP1, inositol 1-phosphate; IP3, inositol 1,4,5-triphosphate; IP2, inositol 1,4-biphosphate; mAb, monoclonal antibody; NE, norepinephrine; PHX, partial hepatectomy; PIP2, phosphatidylinositol; PLC, phospholipase C; TGF-beta, transforming growth factor beta.


CONTROL
8 HR
16 HR
24 HR
48 HR
72 HR
96 HR
120 HR
fmol bound / 10^5 cells
nM [^{3}H]–Prazosin

MATERIALS AND METHODS

Adult male Fischer 344 rats (150–200 g) were obtained through the Animal Genetics and Production Branch of the National Cancer Institute, Bethesda, MD; [^{3}H]-prazosin and [^{125}I]-sodium iodide were purchased from Amersham Corporation, Arlington Heights, IL, and [^{3}H]-myo-inositol was purchased from American Radiolabeled Chemicals, St. Louis, MO. Other chemicals, drugs, and hormones, except where noted, were from Sigma Chemical Company, St. Louis, MO.

Animal surgery and cell and tissue preparations

Two-thirds partial hepatectomies were performed under light ether anaesthesia as described by Higgins and Anderson (1933). All were performed between 7 and 9 A.M. to avoid diurnal variations in responses.

For alpha-1 receptor binding experiments and for assays of NE-stimulated inositol phosphate (IP) release, viable cells were isolated from control or regenerating livers by a modification of the collagenase perfusion technique of Seglen (1976) as previously described (Cruise and Michalopoulos, 1985).

For measurements of *ras* p21 content, animals were killed by decapitation and livers were quickly removed to ice-cold buffer for homogenization as described below.

Membrane preparations and protein extracts

For p21 assays, a crude membrane fraction was obtained by homogenization of liver tissue in ice-cold buffer containing 50 mM Tris-HCl, 1 mM EGTA, 250 mM sucrose, pH 8.0. After centrifugation at 4,300g, the supernatant was saved and the pellet was rehomogenized and recentrifuged. Membranes were pelleted from the pooled supernatants at 37,000g. These were washed and resuspended in 50 mM Tris, 1.0 mM $MgCl_2$, 1.0 mM EGTA, pH 7.4, and sonicated in the above buffer containing 0.05% Tween-20 and then clarified by centrifugation at 10,000g to obtain protein extracts for liquid competition assays as described by Horan Hind et al. (1987). Extracts were stored under liquid nitrogen until assayed. Protein content of samples was determined by the method of Bradford (1976) by using Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, CA).

Alpha-1 receptor binding

Freshly isolated hepatocytes (500,000–1,000,000 cells) were incubated in the following binding buffer (30 mM HEPES, 0.2% BSA, 0.14 M NaCl, 6.7 mM KCl, 100 μM pargyline, pH 7.4) with 0.1–2.0 nM [^{3}H]-prazosin (65–85 Ci/mmol) in a final volume of 0.5 ml. Nonspecific binding (36.5 ± 2.5% of total bound) was determined at all prazosin concentrations by the inclusion of 10 μM phentolamine (gift of Ciba-Geigy Corporation, Suffern, NY). Suspensions were incubated for 30 min at 37°C with constant gentle shaking, and the incubation terminated by rapid filtration and washing with HEPES buffer containing 0.02% BSA. Radioactivity associated with the glass fiber filters (GF/C, Whatman, Ltd., Maidstone, England) was counted by liquid scintillation. Total binding at each concentration was determined in triplicate incubations; nonspecific binding at each concentration was determined in duplicate incubations; n = 3–5 independent experiments per time point examined.

Inositol phosphate (IP) production

Freshly isolated hepatocytes (approximately 40,000,000 cells) were preincubated for 90 min at 37°C with 50 μCi/ml [^{3}H]-myo-inositol (15 Ci/mmol) in 1.0 ml of buffer (10 mM HEPES, 0.14 M NaCl, 6.7 mM KCl, 1.3 mM $CaCl_2$, pH 7.4). Cells were washed to remove unincorporated inositol, resuspended in buffer with 20 mM $LiCl_2$, and incubated for a further 10 min.

Aliquots of cells were then incubated in triplicate for 10 min with 0.01–100 μM NE in $LiCl_2$ buffer (total volume = 220 μl). The reactions were terminated by the addition of 2 ml chloroform:methanol (1:1) and the samples were stored at −20°C. Tritiated, water-soluble IPs formed were separated by anion exchange chromatography as described by Berridge (1983). Peaks representing IP1, IP2, and IP3 were eluted together and counted by liquid scintillation.

ras p21 immunoassay

Affinity-purified pan-reactive rat monoclonal antibody Y13-259 (Furth et al., 1982) was radiolabeled by using chloramine-T and [^{125}I]-sodium iodide. Free iodide was separated from IgG by microcolumn gel centrifugation over Sephadex G-25 (fine) (Parkinson et al., 1981). Specific activity of the mAb was 3,000–8,000 cpm/ng.

Liquid competition

These assays were performed essentially as described by Horan Hand et al. (1987). Briefly, dilutions of detergent extracts from hepatic membranes in Tris buffer containing 0.05% Tween-20 were incubated with 75–100 ng/ml of [^{125}I]-Y13-259 mAb in microwell plates. As internal calibration standards, dilutions of purified recombinant *ras* p21 (kindly provided by Dr. Ray Sweet of Smith, Kline and French Labs, Swedeland, PA) were assayed in parallel. After 1 hr at 25°C, aliquots of these incubations were removed in duplicate to solid-phase "detection" microwell plates prepared by drying approximately 50 μg/well of a protein extract of cells producing v-Ha-*ras* p21 (kind gift of Dr. Steven Strom). The detection plates were incubated overnight at 4°C. The wells were then washed three times in assay buffer, cut from the plates, and counted in a Packard gamma counter.

Fig. 1. Alpha-1-adrenergic receptor binding after partial hepatectomy. Specific binding of [^{3}H]-prazosin to intact hepatocytes isolated from control and regenerating livers at 8, 16, 24, 48, 72, 96, and 120 hr after PHX. No significant change in binding capacity was observed until 48–72 hr after PHX, by which time the number of alpha-1 receptor sites was reduced to 30% of controls. N = 3–5 experiments per point, triplicate samples.

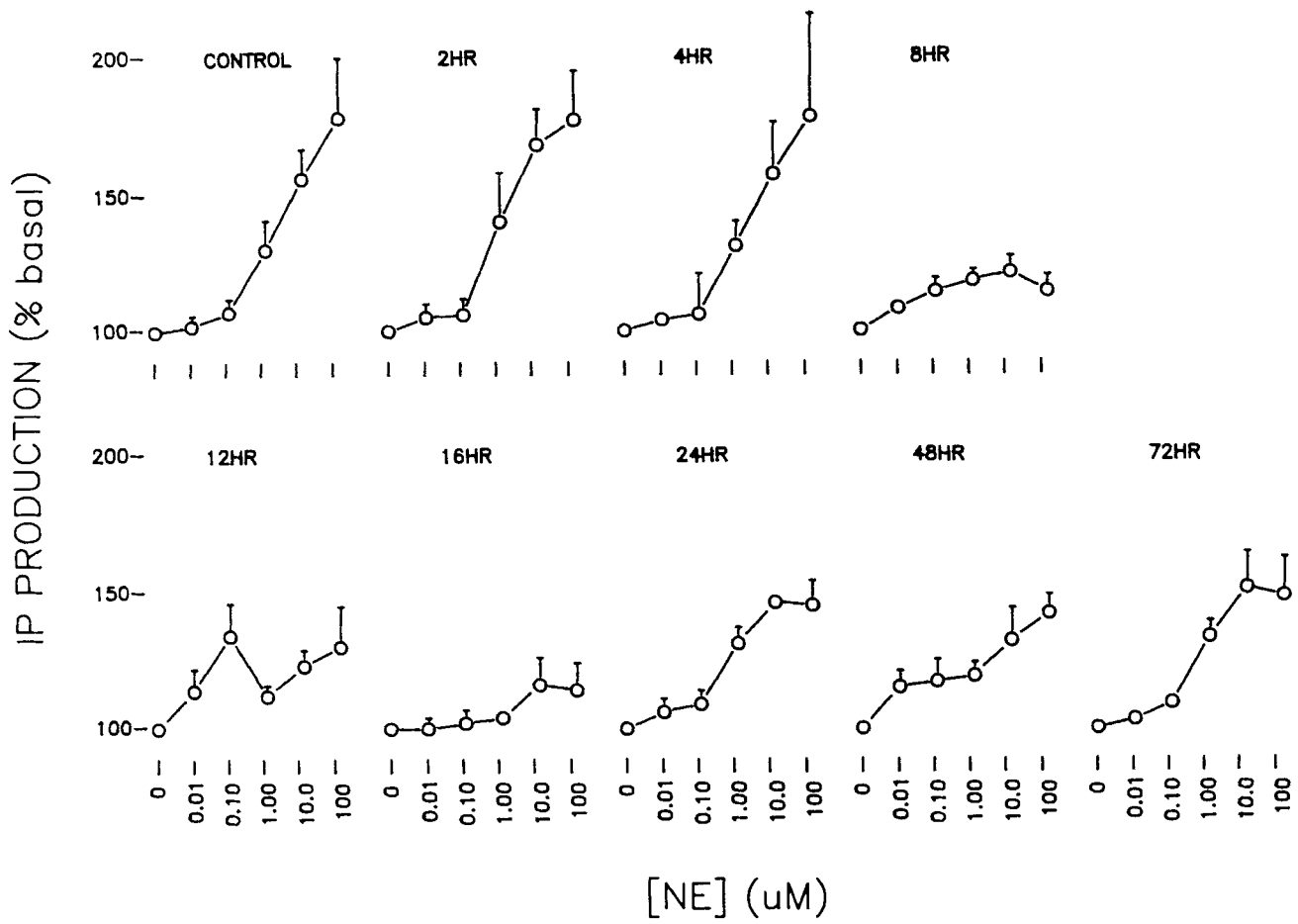


Fig. 2. Stimulation of IP production by norepinephrine after partial hepatectomy. Cells isolated from control and regenerating rat livers were prelabeled by incubation with [^{3}H]-myo-inositol and then incubated for 10 min with NE at concentrations ranging from 0.01 to 100 μM. Labeled, water-soluble inositol phosphates (IPs) formed were separated by ion-exchange chromatography and quantitated by liquid scintillation. Data are expressed relative to basal IP production in cells unsupplemented by NE. Between 8 and 16 hr after PHX, the dose-dependent stimulation of IP generation by NE was lost. By 24 hr, the alpha-1 receptor was again coupled to IP production. N = 2–8 experiments per point, triplicate samples.

RESULTS

Alpha-1 receptors and IP generation

The specific binding of [^{3}H]-prazosin to freshly isolated rat hepatocytes is illustrated in Figure 1. Hepatocytes were isolated from control rats and from animals at different times after partial hepatectomy (PHX). Surface binding capacity indicated the presence of approximately 50,000–60,000 sites per hepatocyte (8–10 fmol/100,000 cells). Binding capacity did not change during the first 24 hr after PHX. Additional saturation curves for 2, 4, and 12 hr also showed no significant changes (data not shown). At 48 and 72 hr after PHX, alpha-1 binding to hepatocytes was decreased, reaching 30% of control by 72 hr. LIGAND analysis of the binding data estimated Bmax from Scatchard plots at 72 hr to be 3.71 ± 0.65 fmol/100,000 cells, compared to 12.95 ± 3.76 fmol/100,000 control cells. No significant changes in kd were noted (control = 0.44 ± 0.12 nM; 72 hr = 0.41 ± 0.25 nM). This loss of binding sites is correlated with the reported loss of alpha-1 mediation of agonist-stimulated glycogenolysis, gluconeogenesis, and ureagenesis 3 days after PHX (Huerta-Bahena et al., 1983).

While no change was observed at early time points in receptor binding, Figure 2 demonstrates that the coupling of receptor occupancy by NE to the generation of IPs is lost between 8 and 16 hr after PHX. After 24 hr, the alpha-1 adrenergic receptor was again coupled to phosphatidylinositol breakdown and the generation of IP. The maximum response to NE was somewhat diminished in magnitude. This may be related to the reduced number of alpha-1 sites after 24 hr.

p21 in regenerating liver

A sensitive radioimmunoassay developed by Horan Hand et al. (1987) was used to estimate *ras* proto-oncogene product in regenerating liver membranes. Detergent extracts of liver membrane fractions served as the source of competing antigen, which was assayed for its ability to absorb [^{125}I]-Y13-259 from solution. Unabsorbed mAb was detected by subsequent incubation with a p21-rich extract of transformed cells dried to a

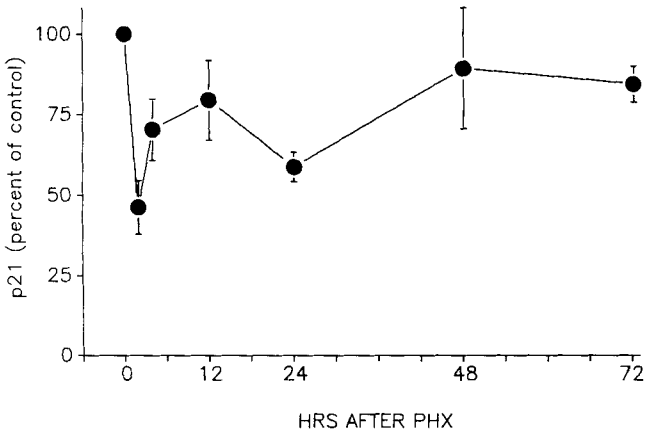


Fig. 3. Liquid competition radioimmunoassay of p21 in detergent extracts of regenerating liver membranes. Protein extracts were made from crude liver membrane preparations. These were obtained from liver lobes taken at the time of hepatectomy (control) and at various times during regeneration. Dilutions of these extracts were assayed as described in Materials and Methods for the ability to compete for binding of [^{125}I]-Y13-259. EC50 values were estimated for each preparation, and regenerating extracts were compared to control extracts from the same animals. Data are expressed as percent of control p21 competing activity. Extractable *ras* p21 fell dramatically within 2 hr of PHX, to 46.2 ± 8.4% of control levels. By 4 hr, membrane p21 had reached 70.3 ± 9.5%. N = 3–4 experiments per time point, duplicate samples.

solid support and was expressed as a percentage of that detected when no competitor was used. Comparing the EC50 values of competition with that obtained by using purified recombinant p21 from *E. coli* as a competitor allowed us to estimate the actual p21 content of our protein extracts. The mean p21 level in control liver membrane extracts was 38 ± 3 pg/μg protein. To control for variations in p21 content between animals, we compared extracts from regenerating liver to extracts made from the same animals' tissue taken at the time of PHX (Fig. 3). Levels of p21 in extracts fell rapidly after PHX, to less than 50% of controls by 2 hr. At 24 hr, membrane extracts contained 60% of their normal extractable p21, and by 72 hr, membrane p21 levels were approaching prehepatectomy controls. The presence or absence of protease inhibitor did not affect the loss of immunoreactivity from membrane fractions (data not shown).

Thompson et al. (1986) did not find this decrease in p21 levels after PHX, but instead found a slight increase in immunoprecipitable protein at 48–72 hr, following an increase in *ras* mRNA at 24 hr (also seen by Hseih et al., 1988). They measured p21 in detergent extracts made from whole liver, rather than from membrane fractions (which are relatively enriched in the mature membrane-associated form of this protein). Figure 4 illustrates that when whole liver extracts were used as the source of competing antigen in our assay, the drop in p21 could not be observed. Shih et al. (1982) have reported that the mature p21 protein is present in whole tissue extracts and cytosol at concentrations too low to be accurately measured, and that the primary source of immunoreactivity in such preparations is its cytoplasmic precursor form, pro-p21. Only in membrane fractions could the mature form be found. While we did not detect any significant increase in cytosolic competing activity paralleling the decreased membrane p21, as we might expect if mature p21 was being translocated to the cytoplasmic fraction (data not shown), it is possible that such changes would be masked by a great dilution of p21 in that fraction.

DISCUSSION

The role of adrenergic agents and their receptors in the modulation of liver regeneration following partial resection has been of interest to many investigators over a number of years (for review, see Morley and Royse, 1981). Regenerative DNA synthesis begins between 16 and 20 hr after PHX (Grisham, 1962), so any early signals should occur before this time. While surface binding of alpha-1 adrenergic receptors remains unchanged, the coupling of these receptors to the breakdown of membrane inositol phospholipids is lost between 8 and 16 hr. If alpha-1 effects on growth are mediated through this breakdown, by 8 hr the "message" might already have been transmitted, and a desensitization of receptors may have begun. Alternatively, the *uncoupled* receptor may be the mediator of interest. Vasopressin and angiotensin II stimulate more breakdown of PIP_2 in hepatocytes than does NE, but they have only weak effects on DNA synthesis in vitro (Cruise et al., 1988). In addition, NE has been demonstrated to overcome growth inhibition by TGF-beta in hepatocytes, via the alpha-1 receptor (Houck et al., 1988). Not only are vasopressin and angiotensin II again only weakly active in this assay, but NE added to hepatocytes isolated from 12 hr regenerating livers (when alpha-1 receptors are uncoupled) is even *more* potent in antagonizing TGF-beta than in normal cells (Houck et al., submitted).

Because changes in expression of the *ras* proto-oncogene typically occur concurrently with DNA synthesis, it has not been considered important in *early* growth signal transduction, despite the gene product's membrane location and G-protein nature. Our data demonstrate that significant changes in immunoreactive protein occur at the membrane within 2 hr of PHX. If the loss of mature p21 from its membrane location alters guanine nucleotide regulation of some receptor or effector molecule, *ras* may yet be involved in growth signaling. The fall in membrane p21 precedes the observed uncoupling of alpha-1 receptors, but additional studies are needed to reveal whether there is more than a temporal association between these events.

Later in regeneration, at 48–72 hr, a down-regulation of alpha-1 adrenergic receptors was observed. The loss of binding correlates with the loss of alpha-1- and the rise of beta-adrenergic control of glycogenolysis, gluconeogenesis, and ureagenesis (Huerta-Bahena et al., 1983). The loss of binding is similar to that found by Aggerbeck et al. during the regenerative response to cholestasis (1983). Other investigators have not found this down-regulation. Plasma membrane preparations from female rats did not demonstrate reduced binding capacity at 72 hr after PHX (Huerta-Bahena et al., 1983), while in crude particulate fractions from male rat liver a 35% lowering of [^{3}H]-prazosin binding has been reported at 18–24 hr (Sandnes et al., 1986). Differences in methodology (whole cells vs. membrane preparations) and the significant sex differences in he-

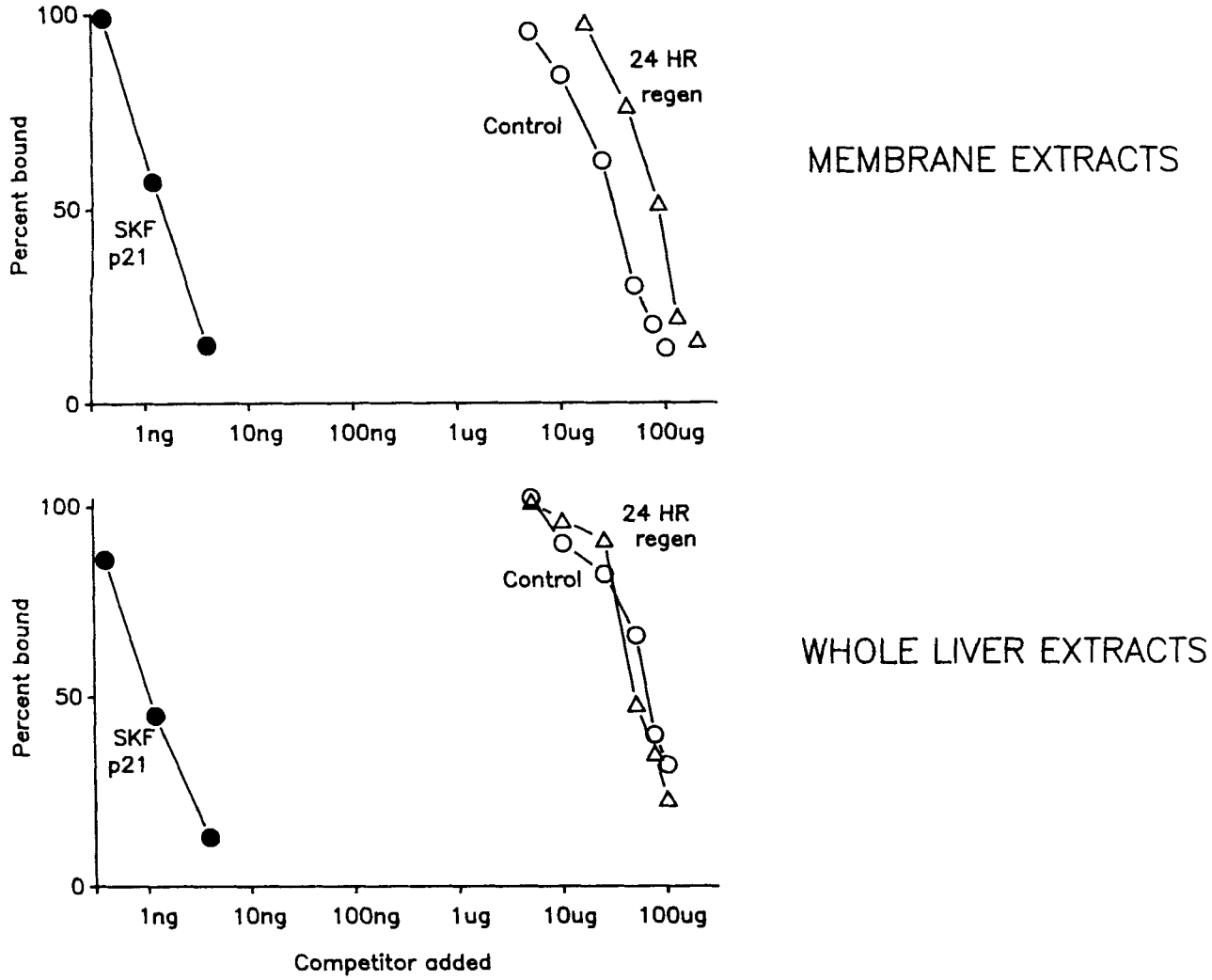


Fig. 4. Liquid competition assay from p21 in membranes vs. whole liver extracts. Protein extracts were obtained from either control or 24 hr regenerating livers. When extracts were made from membrane fractions, regenerating extracts contained less p21, as demonstrated by the higher concentrations needed to compete, relative to control preparations. However, when made from whole liver, extracts from control and regenerating liver were not appreciably different in p21 concentration. By comparison with purified p21, the 24 hr membrane extract contained 19 pg p21/μg membrane, compared to 48 pg/μg in the control. The comparable values for the whole liver extract were 22 and 17 pg/μg. Competition assays were performed as described in Materials and Methods; data are from representative experiments.

patic adrenergic receptor pathways in the rat (Studer and Borle, 1984) may account for the lack of agreement with these data.

We demonstrated a loss of anti-p21 antibody binding in liver membranes soon after hepatectomy. An initial drop in p21 content by 2 hr was followed by a partial restoration and a subsequent decrease between 12 and 24 hr. After 24 hr, p21 content increased toward control membrane levels.

It is intriguing to speculate on a role for the *ras* protooncogene product in the events that follow liver resection. Although models have been proposed that cast p21 as a G-protein regulating PLC breakdown of inositol phospholipids, other evidence points to a function more distal to phospholipid breakdown (Yu et al., 1988), and the effects of overexpression of cellular and viral *ras* genes include both the enhancement and reduction of sensitivity to receptor stimulation of this effect (Wakelam et al., 1986; Parries et al., 1987; Olinger and Gorman, 1988). The use of combinations of additional antibodies should allow us to examine relative changes in the three types of *ras* p21s (Ha-, Ki-, and N-); as a panreactive antibody, Y13-259 presumably detects all populations of p21 proteins.

Future studies will examine the mechanism by which immunoreactive p21 is lost from membrane extracts early after PHX. Initial screening of cytoplasmic extracts suggests that the molecule is not simply translocated away from its membrane location, but the high dilution of p21 in cytosol may require extensive concentration of these extracts for detection. The protein itself may be altered or degraded such that it is no longer recognized by the monoclonal antibody we used (Y13-259). Alternatively, the recently discovered GAP

protein activity of cytosol is apparently prevented from activating N- or H-*ras* p21 by preincubation of the proto-oncogene products with Y13-259 (Adari et al., 1988); it is possible that the association of p21 with a membrane or cytoplasmic component such as GAP might reduce its recognition by this antibody.

Whatever their causes, our data demonstrate that *ras* p21 proteins and alpha-1-adrenergic receptors undergo a complex pattern of changes during the early stages of liver regeneration and may provide clues to the mechanisms which trigger hepatic DNA synthesis.

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