IP receptor agonist-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes: possible involvement of endogenous transforming growth factor-α

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Abstract. To elucidate the mechanism of action of prostaglandin I_2 (PGI_2) and carbaprostacyclin we studied their effect on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. Hepatocyte parenchymal cells, maintained in a serum-free, defined medium, synthesized DNA and proliferated in the presence of PGI_2 (10^{-8} M) or carbaprostacyclin (10^{-9} M) in a time- and dose-dependent manner. PGI_2 was less potent than carbaprostacyclin in stimulating hepatocyte mitogenesis. These effects of PGI_2 and carbaprostacyclin were abolished by treatment with a specific IP receptor antagonist, CAY10441 (10^{-9} ~10^{-7} M), and by the thromboxane A_2 receptor agonist, U46619 (10^{-7} M). Hepatocyte mitogenesis induced by the IP receptor agonists was almost completely blocked by specific inhibitors of growth-related signal transducers such as AG1478 (5×10^{-7} M), LY294002 (10^{-7} M), PD98059 (10^{-6} M), and rapamycin (10 ng/ml). In addition, PGI_2 or carbaprostacyclin significantly increased the kinase activity of a (p175 kDa) receptor tyrosine kinase and the phosphorylation of p42 kDa mitogen-activated protein (MAP) kinase. Addition of a monoclonal antibody against transforming growth factor (TGF)-α, but not insulin-like growth factor-I, to the culture dose-dependently inhibited the PGI_2- or carbaprostacyclin-induced hepatocyte mitogenesis. These results suggest that the IP receptor agonist-induced hepatocyte mitogenesis is mediated by autocrine secretion of TGF-α followed by activation of a receptor tyrosine kinase/MAP kinase pathway.

Keywords: DNA synthesis, proliferation (cultured hepatocyte), prostaglandin I_2, transforming growth factor-α, signal transduction

Introduction

Prostaglandins (PGs) have diverse biological functions as chemical mediators for the maintenance of local homeostasis in nearly all mammalian tissues, including regulatory functions linked to inflammation, platelet aggregation, and contraction of smooth muscles (1). In addition, prostaglandins are also reported to be involved in liver regeneration. Thus prostaglandin E_2 (PGE_2) increases in rat liver after partial hepatectomy. Furthermore, indomethacin both inhibits DNA synthesis in regenerating liver and prevents increases in PGE_2 (2-6).

We have previously shown that each prostaglandin from a different series can stimulate DNA synthesis and proliferation in serum-free primary cultures of adult rat hepatocytes (7). These findings were unexpected since prostaglandins are regarded as co-mitogenic growth factors that enhance the effects of primary mitogens such as insulin and EGF (8, 9). Among these prostaglandins tested the PGI_2 receptor (IP receptor) agonist was of particular interest, as, to date there has been almost no report of its involvement in the stimulation of DNA synthesis and proliferation of primary cultures of adult rat hepatocytes.

PGI_2 (prostacyclin) was isolated from eluates of the stomach fundus and the vascular endothelium, along with prostaglandin endoperoxides. It is well known that PGI_2 is synthesized mainly by the vascular endothelium

and that it is a powerful vascular vasodilator and inhibitor of platelet aggregation (10). In contrast, thromboxane A_2 has antagonistic properties (vasoconstriction, aggregation of platelets) and opposes the actions of PGI_2 on platelets (11). However, relatively little is known about IP receptor effector mechanisms during liver regeneration, although binding sites have been described for PGI_2 (12).

Recently, various types of receptors for PGE_2, $PGF_{2\alpha}$, PGI_2, PGD_2, and thromboxane A_2 (i.e., EP, FP, IP, DP, and TP receptors, respectively) have been characterized using pharmacological and biochemical approaches (1, 13-15). Prostaglandin receptors are members of the superfamily of G protein-coupled receptors that appear to have a single subunit structure containing seven membrane-spanning regions.

The main purpose of the present study was to investigate the growth-promoting effects of IP receptor agonists, including the naturally-occurring PGI_2 agonist and the more stable analog, carbaprostacyclin, on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. In addition, we investigated the role of the IP receptor in mediating the effect of these prostanoids on intracellular signal transduction, as well as possible physiological antagonism by TP receptor agonists. Our results suggest that the IP receptor mediates the proliferative actions of the prostanoids on primary cultured hepatocytes via induction of autocrine secretion of transforming growth factor (TGF)-α, and

subsequent activation of a tyrosine kinase/MAP kinase pathway.

Materials and Methods

Animals

Male Wistar rats weighing 200 - 220 g were obtained from Saitama Experimental Animal Co. (Tokyo, Japan). Adaptation to a light-, humidity- and temperature-controlled room occurred over a minimum 3-day period prior to the start of the experiments. Rats were fed a standard diet and given tap water *ad libitum*. The animals used in this study were handled in accordance with the "Guiding Principle for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society.

Hepatocyte isolation and culture

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). Hepatocytes were isolated from normal livers by a two-step *in situ* collagenase perfusion technique to facilitate disaggregation of the adult rat liver (16, 17). In brief, dispersed hepatocytes were washed three times by slow centrifugation (50 × g, 1 min) of the cell suspension to remove cell debris, damaged cells and non-parenchymal cells. Viability was more than 97% as tested by Trypan blue exclusion. Unless

otherwise indicated, freshly isolated hepatocytes were plated onto collagen-coated plastic culture dishes (Sumitomo Bakelite Co., Tokyo, Japan) at a density of 3.3×10^4 cells/cm^2 (3.0×10^5 cells/35-mm dish), and allowed to attach for 3 h in Williams' medium E containing 5% newborn calf serum, 0.1 nM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.10 μg/ml aprotinin in 5% CO_2 in air at 37°C. The medium was then replaced by aspiration, and the cells were further cultured in serum- and dexamethasone-free Williams' medium E supplemented with prostaglandins such as PGI_2 and carbaprostacyclin. When appropriate, IP receptor agonists with or without CAY10441, SC-51322, U-73122, U-73343, sphingosine, H-89, 2,4-dideoxyadenosine, ionomycin, verapamil, somatostatin or the growth-related signal transduction inhibitors AG1478, LY294002, PD980059, or rapamycin were added at the concentrations indicated in the text.

Measurement of DNA synthesis

Hepatocyte DNA synthesis was assessed by measuring the incorporation of [^{3}H]thymidine into acid-precipitable materials. Briefly, after an initial attachment period of 3 h, the hepatocytes were washed twice with serum-free Williams' medium E and cultured in a medium containing IP receptor agonists with or without agents to be tested for an additional 4 or 21 h. The cells were pulsed with [^{3}H]thymidine (1.0 μCi/well) at 1, 2, 3 or 19

h after a 2-h prostaglandin stimulation followed by 10% trichloroacetic acid precipitation, as described previously (18). [^{3}H]Thymidine incorporation into DNA was counted as counts per min using a scintillation counter and was normalized for cellular protein. Aphidicolin (10 μg/ml) was added to some wells to establish the level of non-replicative DNA synthesis. Hepatocyte protein content was measured by a modified Lowry procedure using bovine serum albumin as the standard (19). Data are expressed as dpm/h/mg cellular protein.

Nuclei counting

The number of nuclei, rather than the number of cells, was counted using a modified version of a previously described procedure (20, 21). Briefly, primary cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (PBS, pH 7.4). Isolated liver cell nuclei were then prepared for quantification by exposure of the cultured hepatocytes to 0.25 ml of citric acid (0.1 M) containing Triton X-100 (0.1%) for 30 min at 37°C. An equal volume of the nuclear suspension was mixed with Trypan blue (0.3%) in PBS (pH 7.4) and the number of nuclei was counted in a hemocytometer.

Determination of receptor tyrosine kinase activity

A 175-kDa protein was identified as the EGF/TGF-α receptor by

immunoblotting with a specific anti-phospho-receptor tyrosine kinase antibody according to the manufacturer's instructions. In brief, hepatocytes were freshly isolated and seeded at a density of 3.3×10^4 cells/cm^2 and cultured in Williams' medium E containing 5% newborn bovine serum. The medium was then replaced by aspiration and the cells were further cultured in serum- and dexamethasone-free Williams' medium E. Cultured hepatocytes were washed once with ice-cold phosphate-buffered saline (pH 7.4) and then 0.2 ml lysis buffer [20 mM Tris buffer, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride] was added. Cell lysates were obtained by scraping the cells in lysis buffer followed by sonication for 3 min. Cell lysates were spun down (3000 × g for 3 min at 4°C), and then denatured in boiling water for 5 min. For immunoblotting analysis, samples of the supernatant (30 μg/lane) were resolved by SDS-polyacrylamide gel electrophoresis, using a 7.5% polyacrylamide resolving gel (22), transferred to PVDF membrane and immunoblotted with the anti-phosphotyrosine antibody PY 20 (23). Blots were developed by enhanced chemiluminescence following incubation with HRP-conjugated secondary antibodies (24). Proteins were quantified by densitometry after the membrane was developed with enhanced chemiluminescence reagent and exposure to Hyperfilm (Kodak). Densitometric analysis was performed using the NIH

image program (ver. 1.6 for Macintosh). The tyrosine kinase activity (autophosphorylation) of the phosphorylated p175 kDa protein (P-p175 kDa) was normalized to that of the total p175 kDa protein. The supernatant protein concentration was determined using the Bio-Rad DC protein assay.

Determination of MAP kinase activity

Phosphorylated (activated) MAP kinase isoforms (p42 and p45) were identified by western blot analysis of cell lysates using a 1:1000 dilution of rabbit polyclonal dual phospho-specific antibodies (1 mg/ml) with HRP-conjugated goat anti-rabbit IgG as a secondary antibody, as previously described (23). Cell lysis and western blotting procedures were carried out as described in the previous section except that 20 μg of the supernatant was applied/lane of a 10% polyacrylamide resolving gel. Phosphorylated MAP kinase (P-42 MAP kinase) activity was normalized to the total MAP kinase activity. The data were calculated in arbitrary units and are expressed as means ± S.E.M. (*$P < 0.05$ when compared to the medium alone). The autodiagram is a representation of three experiments using different cell preparations.

Neutralization of endogenous growth factors

In experiments employing neutralizing antibodies, serum-free primary

cultured hepatocytes were treated with varying concentrations of IP receptor agonists in the presence or absence of monoclonal antibodies against IGF-I or TGF-α (12.5, 25, 50, 75 or 100 ng/ml).

Materials

The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA): aphidicolin, ionomycin, dexamethasone, somatostatin, verapamil hydrochloride, and aprotinin. Monoclonal antibodies against IGF-I and TGF-α, as well as SC-51322 (2-[3-[(2-furanylmethyl)-thiol]-1-oxopropyl]hydrazide), U-73122 (1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino] hexyl]-1H-pyrrol-2, 5-dione), U-73343 (1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino] hexyl]-2, 5-pyrrolidine-dione), sphingosine, 2,4-dideoxyadenosine, H-89 (N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride), AG1478 (2-[4-morpholinyl]-8-phenyl-1(4H)-benzopyran-4-one), LY294002 (N-[3-chlorophenyl]-6,7-dimethoxy-4-quinazolinamine), and rapamycin, were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). CAY 10441 (4,5-dihydro-N-[4-[[4-(1-methylethxy)phenyl] methyl]-1H-imidazol-2-amine) was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). PD98059 (2'-amino-3'-methoxyflavone) was obtained from Calbiochem-Behring (La Jolla, CA, USA). Williams' medium E and

newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical Co. (Freehold, NJ, USA). [Methyl-^{3}H] thymidine (20 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA, USA). All other reagents were of analytical grade.

Statistical analysis

Data are expressed as means ± S.E.M. Group comparisons were made by analysis of variance (ANOVA) for unpaired data followed by post hoc analysis using Dunnett's multiple comparison test. Differences of $P < 0.05$ were considered to be statistically significant.

Results

Time course of induced stimulation of hepatocyte DNA synthesis and proliferation by IP receptor agonists

We first examined the effects of IP receptor agonists on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes in the absence of exogenously added peptide growth factors. When maintained for a short time in culture medium containing PGI_2 (10^{-8} M) or

carbaprostacyclin (10^{-9} M) the hepatic parenchymal cells underwent time-dependent DNA synthesis and proliferation (i.e., an increase in the number of nuclei). The onset of DNA synthesis was first observed about 2.5 h after the addition of PGI_2 or carbaprostacyclin (Fig. 1A), while mitotic activity of the hepatocytes was first observed at about 3.0 h and peaked at 4.0 h (Fig. 1B). Maximal stimulation of hepatocyte DNA synthesis and proliferation by PGI_2 and carbaprostacyclin was approximately 6.0-fold and 1.3-fold, respectively.

Dose-response effects of IP receptor agonists on hepatocyte DNA synthesis and proliferation

We next examined the dose-response relationship between the IP receptor agonists and DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. PGI_2-induced DNA synthesis was dose-dependent and reached a plateau at 10^{-8} M, with a half-maximal effective concentration (ED_{50}) value of 1.0×10^{-9} M (Fig. 2A). Carbaprostacyclin-induced DNA synthesis was also dose-dependent and reached a plateau at 3×10^{-9} M, with an ED_{50} value of 1.8×10^{-10} M (Fig. 2A). Despite the fact that carbaprostacyclin was about one order of magnitude more potent than PGI_2 in stimulating hepatocyte DNA synthesis, the maximal response induced by carbaprostacyclin and PGI_2 was almost the same. The proliferative effect (i.e., increase in the number of nuclei) of these IP receptor agonists

on cultured hepatocytes were very similar to their effects on DNA synthesis (Fig. 2B). In contrast the effect of U46619, a TP receptor agonist, on DNA synthesis and proliferation in cultured hepatocytes was negligible being in the concentration range of 10^{-12} to 10^{-7} M (Figs. 2A and 2B).

Effects of specific IP receptor antagonists, EP receptor antagonists, and TP receptor agonists, on hepatocyte DNA synthesis and proliferation induced by IP receptor agonists

To confirm that IP receptor agonist cellular effects were mediated by IP-receptors we investigated the effect of an IP receptor antagonist (CAY10441), EP_1 receptor antagonist (SC-51322), or U46619 on IP receptor agonist-induced-hepatocyte DNA synthesis and proliferation after 4 h of culture. The DNA synthesis and proliferative effects of PGI_2 (10^{-8} M) and carbaprostacyclin (10^{-9} M) on primary cultured hepatocytes were inhibited by CAY10441 (10^{-9} to 10^{-7} M) in a dose-dependent manner (Figs. 3A and 3B). The effects of the IP receptor agonists on hepatocyte DNA synthesis and proliferation were not inhibited by SC-51322 (10^{-10} to 10^{-7} M). Although addition of U46619 (10^{-7} M) alone did not affect hepatocyte DNA synthesis or proliferation (Figs. 2A and 2B) U46619 did inhibit IP receptor agonist-induced hepatocyte DNA synthesis and proliferation in a dose-dependent manner (Figs. 3A and 3B).

Effects of inhibitors or stimulators of signal-transducers, including adenylate cyclase/protein kinase A and the phospholipase C/Ca^{2+}/protein kinase C pathway, on IP receptor agonist-induced hepatocyte DNA synthesis and proliferation

We pharmacologically investigated the intracellular signal transduction events associated with IP receptor agonist treatment of primary cultured hepatocytes to determine how IP receptor agonists induce hepatocyte DNA synthesis and proliferation. To clarify whether IP receptor agonists stimulate hepatocyte DNA synthesis and proliferation via an adenylate cyclase/protein kinase A pathway, we investigated whether a direct inhibitor of adenylate cyclase, 2,4-dideoxyadenosine, or an inhibitor of protein kinase A, H-89, had inhibitory effects on hepatocyte mitogenesis induced by PGI_2 (10^{-8} M) or carbaprostacyclin (10^{-9} M). Neither 2,4-dideoxyadenosine (10^{-6} M) nor H-89 (10^{-7} M) affected IP receptor agonist-induced hepatocyte DNA synthesis or proliferation (Figs. 4A and 4B), suggesting that adenylate cyclase and protein kinase A may not contribute to hepatocyte mitogenesis induced by these IP receptor agonists. Moreover, 2,4-dideoxyadenosine (10^{-6} M) and H-89 (10^{-7} M), when administered alone, did not significantly influence hepatocyte DNA synthesis or proliferation over 4 h of culture (data not shown).

To characterize a possible involvement of the phospholipase C (PLC)/Ca^{2+}/protein kinase C pathway in IP-receptor-mediated stimulation of

hepatocyte DNA synthesis and proliferation induced by PGI_2 or carbaprostacyclin, we investigated the effect of the specific PLC inhibitor, U-73122, and a protein kinase C inhibitor, sphingosine, on these responses. Addition of U-73122 (10^{-6} M) markedly attenuated PGI_2-(10^{-8} M) and carbaprostacyclin- (10^{-9} M) stimulation of hepatocyte DNA synthesis and proliferation over 4 h of culture (Figs. 4A and 4B). Neither U-73343 (10^{-6} M), a close structural analog of U-73122 with no inhibitory action on PLC, nor sphingosine (10^{-6} M), significantly affected PGI_2 or carbaprostacyclin-induced hepatocyte DNA synthesis or proliferation over 4 h of culture., The inhibitors alone did not produce any significant effects on hepatocyte DNA synthesis or proliferation (data not shown) over the 4-h incubation period.

To determine the possible involvement of Ca^{2+} mobilization in PGI_2- or carbaprostacyclin-induced hepatocyte DNA synthesis and proliferation, the cells were similarly treated with ionomycin, a Ca^{2+} ionophore, for 4 h. Significant potentiation of both PGI_2- and carbaprostacyclin-induced hepatocyte DNA synthesis and proliferation was observed following ionomycin (10^{-7} M)treatment. These findings suggest that PLC-independent agents, that elevate intracellular Ca^{2+} levels, enhance PGI_2 or carbaprostacyclin-induced hepatocyte mitogenesis. Conversely, the ability of PGI_2 and carbaprostacyclin to stimulate hepatocyte DNA synthesis and proliferation was almost completely inhibited by Ca^{2+}

channel blockers such as verapamil (10^{-6} M). In addition, somatostatin (10^{-7} M), which inhibits the release of certain gastrointestinal and pancreatic hormones (presumably by affecting decreases in cytosolic Ca^{2+}), strongly inhibited hepatocyte DNA synthesis and proliferation induced by these IP receptor agonists. However, these inhibitors and stimulators by themselves did not influence hepatocyte DNA synthesis or proliferation over 4 h of culture (data not shown).

Effects of specific inhibitors of growth-related signal-transducers on hepatocyte DNA synthesis and proliferation induced by PGI_2 or carbaprostacyclin

We next investigated whether the mitogenic responses of primary cultured hepatocytes to the IP receptor agonists were mediated by signal transducers such as receptor tyrosine kinases, phosphatidylinositol 3-kinase, mitogen-activated protein (MAP) kinase kinase or ribosomal protein S6 kinase (p70 S6K), by using the corresponding specific inhibitors of these signal transducers: AG1478, LY294002, PD98059, and rapamycin, respectively. As shown in Figs. 5A and 5B, hepatocyte DNA synthesis and proliferation induced by PGI_2 (10^{-8} M) or carbaprostacyclin (10^{-9} M) were almost completely inhibited by AG1478 (5×10^{-7} M), LY294002 (10^{-7} M), PD98059 (10^{-6} M) or rapamycin (10 ng/ml). The inhibitors by themselves did not affect hepatocyte DNA synthesis or proliferation over 4 h of culture.

Effect of specific inhibitors of growth-related signal transducers on receptor tyrosine kinase and MAP kinase activity induced by PGI_2 or carbaprostacyclin

To obtain further support for receptor tyrosine kinase/ MAP kinase mediation of the IP receptor agonist action, we examined the effects of specific inhibitors of growth-related signal transducers on receptor tyrosine kinase and MAP kinase activities induced by IP receptor agonists. Figure 6A shows that PGI_2 (10^{-8} M) or carbaprostacyclin (10^{-9} M) caused an increase in the tyrosine phosphorylation of a 175 kDa protein that peaked, 20 min after addition, at about 3.0-fold (compared with control). When PGI_2 or carbaprostacyclin were added in combination with AG1478 (5×10^{-7} M), AG1478 completely abolished the IP receptor agonist-induced increase in receptor tyrosine kinase activity. In contrast, receptor tyrosine kinase activation induced by IP receptor agonists was not abolished by LY294002 (10^{-7} M), PD98059 (10^{-6} M) or rapamycin (10 ng/ml) treatment. In addition the TP receptor agonist U46619 (10^{-7} M) abolished IP receptor agonist-induced receptor tyrosine kinase activation.

PGI_2 (10^{-8} M) and carbaprostacyclin (10^{-9} M) caused an increase in the phosphorylation of p42 MAP kinase, but not p44 MAP kinase, peaking at about 3.0-fold (compared with control) 20 min after addition (Fig. 6B). When the agonists were added in combination PD98059 (10^{-6} M), PD98059 completely abolished the IP receptor agonist-induced increase in p42 MAP

kinase. Moreover, p42 MAP kinase activation induced by IP receptor agonists was abolished by AG1478 (5×10^{-7} M) or LY294002 (10^{-7} M), but not by rapamycin (10 ng/ml) treatment. In addition, U46619 abolished IP receptor agonist-induced p42 MAP kinase activation.

Effects of monoclonal antibodies against TGF-α or IGF-I on hepatocyte DNA synthesis and proliferation induced by PGI_2 or carbaprostacyclin

The data in Figs. 4 - 6 show that IP receptor agonist-induced hepatocyte DNA synthesis and proliferation are mediated through both the IP receptor/(Gq)/PLC/Ca^{2+} pathway and a receptor tyrosine kinase/MAP kinase cascade. However, how these pathways interact with each other remains to be elucidated. Since prostaglandins mainly act as co-mitogens rather than as mitogens both *in vivo* and *in vitro*, we hypothesized that hepatocyte mitogenesis mediated by the IP receptor agonists might be due to selective induction of secretion of primary mitogens in an autocrine manner. Potential primary mitogenic candidates are TGF-α and IGF-I since hepatocytes express mRNA for TGF-α and IGF-I, and can synthesize and store these primary growth factors (9).

To examine the possibility that TGF-α or IGF-I mediate IP agonist-induced hepatocyte DNA synthesis and proliferation in primary cultures, we examined the effect of addition of neutralizing monoclonal antibodies against TGF-α and IGF-I on these processes. Figure 7 shows

that addition of a neutralizing monoclonal antibody against TGF-α dose-dependently inhibited the growth-promoting effect of PGI_2 (10^{-8} M) and carbaprostacyclin (10^{-9} M) on hepatocyte DNA synthesis and proliferation. The IC_{50} values for these effects on synthesis and proliferation after 4 h of culture were 25 and 35 ng/ml, respectively. In contrast, the DNA synthesis and proliferative effects of IP receptor agonists were not significantly affected by treatment of hepatocytes with various concentrations of a monoclonal antibody against IGF-I (12.5 - 100 ng/ml). These monoclonal antibodies by themselves did not significantly influence hepatocyte DNA synthesis and proliferation over 4 h of culture (data not shown). The results indicate that abrogation of the effects of PGI_2 (10^{-8} M) and carbaprostacyclin (10^{-9} M) was specific for the antibody against TGF-α.

Discussion

We have demonstrated that PGI_2 and a stable analog of PGI_2, carbaprostacyclin, significantly induce DNA synthesis and proliferation in primary cultures of adult rat hepatocytes in the absence of exogenously added primary growth factors (shown in Figs. 1 and 2). The growth-promoting effects of IP receptor agonists are almost completely inhibited by the IP receptor antagonist, CAY 10441. In contrast, the EP_1

receptor subtype-specific antagonist SC-51322 (25) did not affect IP receptor agonist-induced hepatocyte DNA synthesis or proliferation (Fig. 3), confirming that the IP receptor agonists apparently act as complete mitogens via the IP receptor in primary cultures of adult rat hepatocytes. However, postreceptor mechanisms responsible for the proliferative action of the IP receptor agonists, as well as intracellular signal transduction mechanisms, remain to be clarified. Interestingly, it was found that the combination of IP receptor agonists and a TP receptor agonist U46619 showed antagonistic effects on hepatocyte mitogenesis induced by IP receptor agonists.

It has been reported that signal transduction mechanisms that may serve as mediators for prostanoid receptors include 1) stimulation of adenylate cyclase via the Gs protein, 2) inhibition of adenylate cyclase via the Gi protein, 3) stimulation of phosphatidylinositol-phospholipase C (PLC) via Gq and, possibly, 4) elevation of intracellular Ca^{2+} through an phosphatidylinositol-dependent process (1). Although cAMP generation is generally regarded as the sole signal transduction system of IP receptors, there have been several reports that PGI_2 and its analogs cause increases in intracellular Ca^{2+} levels and evoke smooth muscle contraction (26, 27). In addition, IP receptor agonist-induced inositol phosphate breakdown and inositol trisphosphate formation have also been reported in mouse thymus medulla (15). In order to clarify which signaling pathway is involved in

IP-mediated hepatocyte mitogenesis, we pharmacologically investigated which signal transduction pathways are mediated by IP receptor stimulation. As shown in Fig. 4, IP receptor agonist-induced hepatocyte mitogenesis was inhibited by the PLC inhibitor, U-73122 (28), but not by its inactive analog U-73343. Therefore, a primary mechanism by which hepatocyte IP receptors in primary cultures mediate PGI_2 or carbaprostacyclin-induced hepatocyte DNA synthesis and proliferation is likely to be via stimulation of phosphatidylinositol-phospholipase C and increased Ca^{2+} mobilization. If this is indeed true, then these responses may also be stimulated by the Ca^{2+}-ionophore ionomycin, which increases Ca^{2+} influx into cultured hepatocytes. Conversely, the Ca^{2+} channel blocker verapamil should inhibit DNA synthesis and proliferation induced by IP receptor agonists. Indeed both ionomycin and verapamil had these effects in our experiments as shown in Fig. 4. Therefore, activation of the PLC/Ca^{2+} pathway by IP receptor agonists appears to be essential for triggering hepatocyte DNA synthesis and proliferation.

On the other hand, it appears unlikely that (Gq)/PLC/protein kinase C, or adenylate cyclase/protein kinase A, contribute to IP receptor-agonist induced hepatocyte mitogenesis. Thus neither an inhibitor of protein kinase C, sphingosine (29), the direct inhibitor of adenylate cyclase, 2,4-dideoxyadenosine (30), nor the inhibitor of protein kinase A, H-89 (31), affected DNA synthesis or proliferation (Fig. 4).

Specific inhibitors of growth-related signal transducers, an EGF-receptor tyrosine kinase inhibitor AG1478 (32), a phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 (33), a MAP kinase kinase inhibitor PD98059 (34), and a P70 S6 kinase inhibitor rapamycin (35) attenuated IP receptor agonist-stimulated hepatocyte DNA synthesis and proliferation (Fig. 5). Therefore, mitogenic signaling through the IP receptor pathway also requires activation of a receptor tyrosine kinase, PI3K, MAP kinase kinase, and p70S6K (Fig. 5). Although both the IP receptor/(Gq)/PLC/Ca^{2+} signaling pathway and the tyrosine kinase/MAP kinase signaling pathway are critically involved in IP receptor agonist-induced hepatocyte DNA synthesis and proliferation, the links between these signaling pathways have not been characterized in detail. Moreover, there is currently little evidence that IP receptor/(Gq)/PLC/Ca^{2+} pathways directly stimulate (or phosphorylate) elements of the tyrosine kinase/MAP kinase signaling pathway to induce cell proliferation (36-38).

In a previous study, we demonstrated that prostaglandin E_2 induced hepatocyte DNA synthesis and proliferation through autocrine secretion of TGF-α, which, in turn, stimulated hepatocyte mitogenesis (24). Therefore, we hypothesized that the IP receptor/(Gq)/PLC/Ca^{2+} pathway might significantly stimulate secretion of a specific mitogen by cultured hepatocytes in an autocrine manner thereby inducing hepatocyte DNA synthesis and proliferation through stimulation of a downstream receptor

tyrosine kinase/MAP kinase pathway. Mitogens that could fulfill this requirement are TGF-α and IGF-I. Both TGF-α and IGF-I are reported to be primary mitogens that are synthesized and stored in parenchymal hepatocytes, and are highly active growth factors that stimulate hepatocyte DNA synthesis and proliferation (5, 39-42). Therefore, we examined the effects of monoclonal antibodies against the putative growth factors TGF-α and IGF-I, on IP receptor agonist-induced hepatocyte DNA synthesis and proliferation. As shown in Fig. 6, both PGI_2- and carbaprostacyclin-induced hepatocyte DNA synthesis and proliferation were almost completely inhibited by the monoclonal antibody against TGF-α, but not by that against IGF-I. In addition, monoclonal antibodies against EGF and HGF (12.5 - 100 ng/ml) did not significantly affect hepatocyte DNA synthesis and proliferation induced by IP receptor agonists (data not shown). Therefore, we suggest that the cytokine TGF-α is stored within the parenchymal hepatocytes and its secretion to the extracellular medium is triggered via IP receptor stimulation. Based on the above pharmacological analysis, we propose that the IP receptor is linked to a G-protein (possibly Gq) and stimulates the activity of PLC. This causes an increase in intracellular Ca^{2+} levels, which induces TGF-α secretion in an autocrine manner. The secreted TGF-α can induce hepatocyte DNA synthesis and proliferation via the TGF-α receptor tyrosine kinase/phosphatidylinositol 3 kinase/p42 MAP kinase/p70 S6K pathway.

In conclusion, we provide evidence that IP receptor agonists are powerful initiators of DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. Conversely, a thromboxane A_2 receptor agonist opposes hepatocyte mitogenesis induced by IP receptor agonists. Based on the antibody neutralizing experiments the IP receptor-dependent autocrine secretion of TGF-α is an essential step in the stimulation of DNA synthesis and proliferation, which is mediated by a receptor tyrosine kinase/MAP kinase pathway. IP receptor agonist-induced TGF secretion and the mechanisms by which it functions and is regulated are currently under investigation.

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Figure Legends

Fig. 1 Time course of the induced stimulation of hepatocyte DNA synthesis and proliferation by IP receptor agonists. Freshly isolated hepatocytes were cultured in Williams' medium E containing 5% newborn bovine serum, 0.1 nM dexamethasone, 0.10 μg/ml aprotinin, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at a cell density of 3.3×10^4 cell/cm^2. After a 3 h-attachment period (time = 0), the medium was rapidly replaced with serum- and dexamethasone-free Williams' medium E with or without 10^{-8} M PGI_2 or 10^{-9} M carbaprostacyclin, and cultured for the indicated times. Hepatocyte DNA synthesis and proliferation were determined as described in Materials and Methods. The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei compared to the control culture (B). The results are expressed as the means ± S.E.M. of three experiments. *$P < 0.05$, **$p < 0.01$ compared with the respective control.

Fig. 2 Dose-response effects of IP receptor agonists on hepatocyte DNA synthesis and proliferation. Freshly isolated hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. After the medium change, the hepatocytes were cultured with various concentrations of PGI_2, carbaprostacyclin, or U46619 for a further 4 h. The

rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei compared with the control culture (B). The results are expressed as means ± S.E.M. of three independent experiments. **P < 0.01 compared with the control (medium alone).

Fig. 3 Effects of CAY10441, SC-51322 and U46619 on hepatocyte DNA synthesis and proliferation induced by IP receptor agonists. Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. After the medium change, the hepatocytes were cultured with 10^{-8} M PGI_2 or 10^{-9} M carbaprostacyclin in the presence or absence of CAY 10441 (10^{-10} to 10^{-7} M), SC-51322 (10^{-10} to 10^{-7} M) or U46619 (10^{-10} to 10^{-7} M) for 4 h. The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei compared with the control culture (B). The results are expressed as means ± S.E.M. of three independent experiments. *P < 0.05, **p < 0.01 compared with the respective control.

Fig. 4 Effects of inhibitors of the adenylate cyclase/protein kinase A pathway or the phospholipase C/protein kinase C pathway on hepatocyte DNA synthesis and proliferation induced by PGI_2 or carbaprostacyclin.

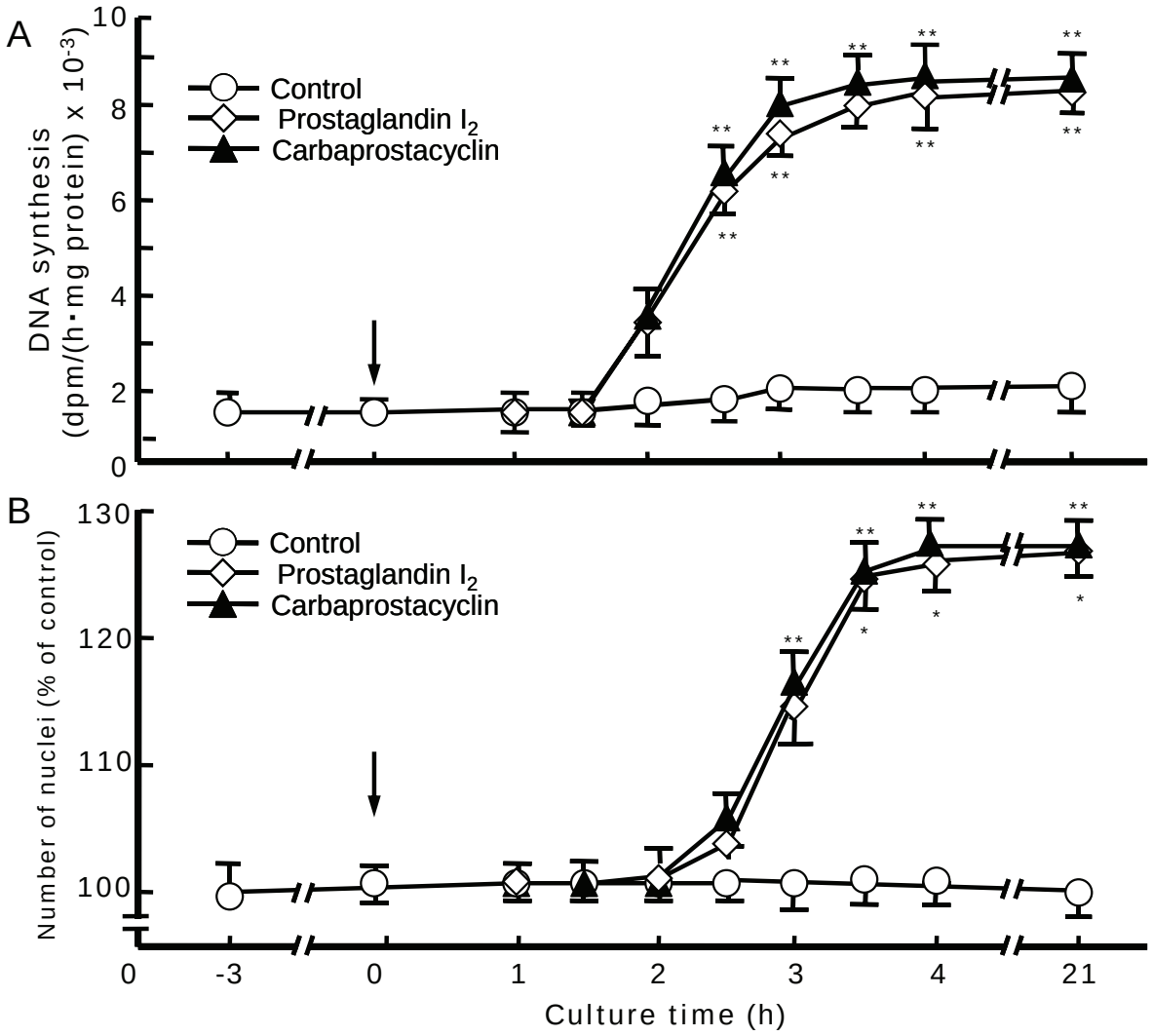
Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. Specific inhibitors or an agonist were added together with 10^{-8} M PGI_2 or 10^{-9} M carbaprostacyclin immediately after the medium change and the hepatocytes were cultured for a further 4 h. Concentrations were as follows: 2,4-dideoxyadenosine, 10^{-6} M; H-89, 10^{-7} M; U-73122, 10^{-6} M; U-73343, 10^{-6} M; sphingosine, 10^{-6} M; verapamil, 10^{-6} M; somatostatin 10^{-7} M; ionomycin, 10^{-7} M. The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei compared with the control culture (B). The results are expressed as means ± S.E.M. of three independent experiments. *P < 0.05, **p < 0.01 compared with the respective control.

Fig. 5 Effects of specific inhibitors of growth-related signal transducers on hepatocyte DNA synthesis and proliferation induced by PGI_2 or carbaprostacyclin. Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. Specific signal-transducer inhibitors were added without or with 10^{-8} M PGI_2 or 10^{-9} M carbaprostacyclin immediately after the medium change, and the cells were cultured for a further 4 h. The concentrations were as follows: AG1478, 5 x 10^{-7} M; LY294002, 10^{-7} M; PD98059, 10^{-6} M; and rapamycin, 10 ng/ml. The rate of hepatocyte DNA synthesis is expressed as dpm/mg

protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei compared with the control culture (B). The results are expressed as means ± S.E.M. of three independent experiments. *P < 0.05, **p < 0.01 compared with the respective control.

Fig. 6 Effect of specific inhibitors of growth-related signal transducers on receptor tyrosine kinase and MAP kinase activity induced by PGI_2 or carbaprostacyclin. Freshly isolated hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. After a medium change, hepatocytes were cultured with IP receptor agonists with or without specific inhibitors of signal transducers for 3 or 5 min. Receptor tyrosine kinase and MAP kinase activities were determined by Western blotting analysis as described in the Materials and Methods. Phosphorylated receptor tyrosine kinase (P-p175 kDa) and total receptor tyrosine kinase protein (p175 kDa) (A). Phosphorylated MAP kinase isoforms (P-p42 kDa, P-p44 kDa) and total MAP kinase protein (p42 kDa, p44 kDa) (B). Concentrations were as follows: PGI_2, 10^{-8} M; carbaprostacyclin, 10^{-9} M; somatostatin, 10^{-6} M; AG1478, 10^{-7} M; LY294002, 10^{-7} M; and PD98059, 10^{-6} M. The results are expressed as the means ± S.E.M. of three different experiments. *P<0.05, **p<0.01 compared with respective controls.

Fig. 7 Effects of monoclonal antibodies against TGF-α or IGF-I on IP receptor agonist-induced hepatocyte DNA synthesis and proliferation. Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. After the medium change, hepatocytes were treated with 10^{-8} M PGI_2 or 10^{-9} M carbaprostacyclin for 4 h in the presence or absence of TGF-α-neutralizing antibody or IGF-I-neutralizing antibody (2.5 - 100 ng/ml). The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei compared with the control culture (B). The results are expressed as means ± S.E.M. of three independent experiments. *$P < 0.05$, **$p < 0.01$ compared with the respective control.



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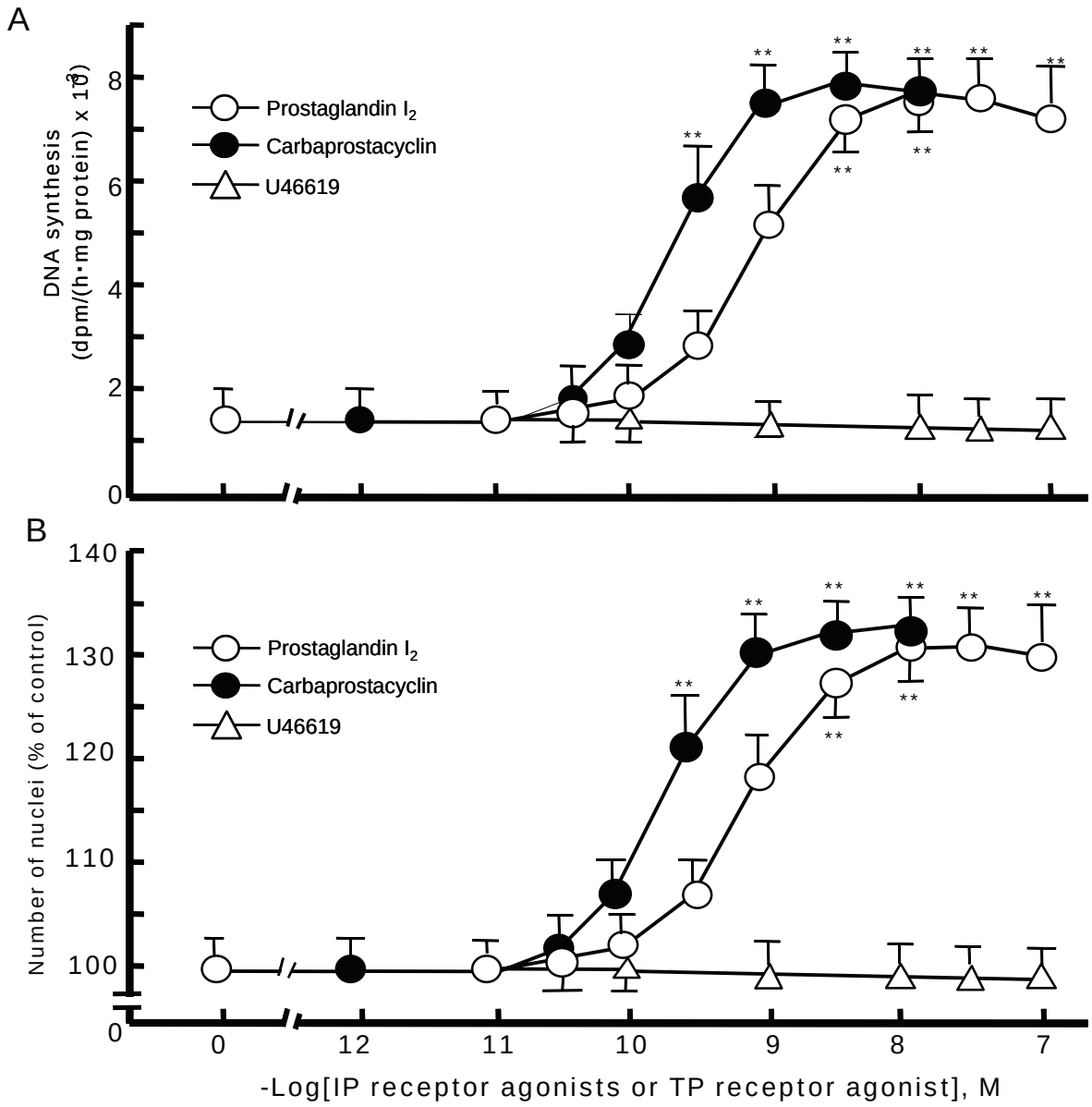

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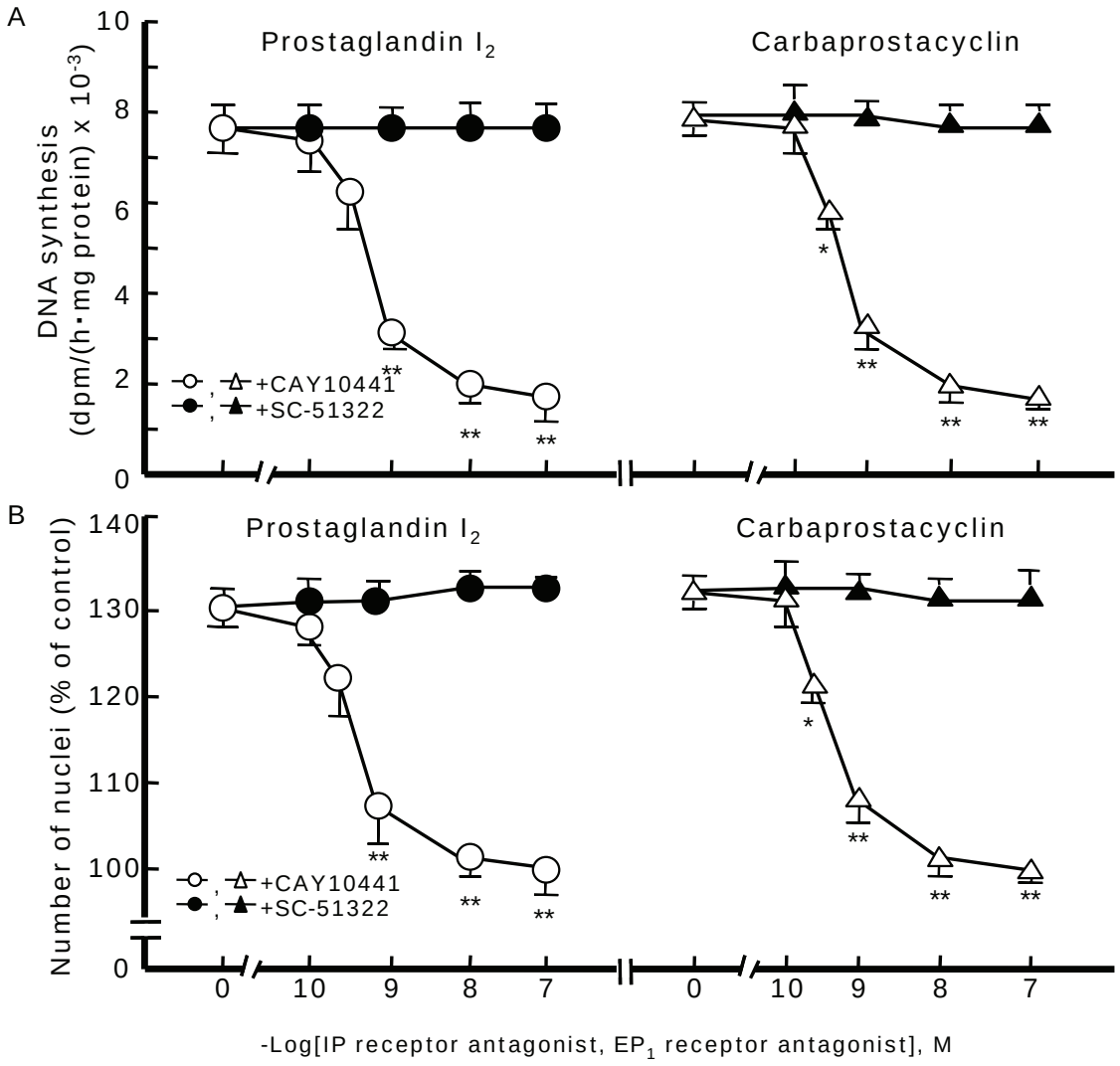

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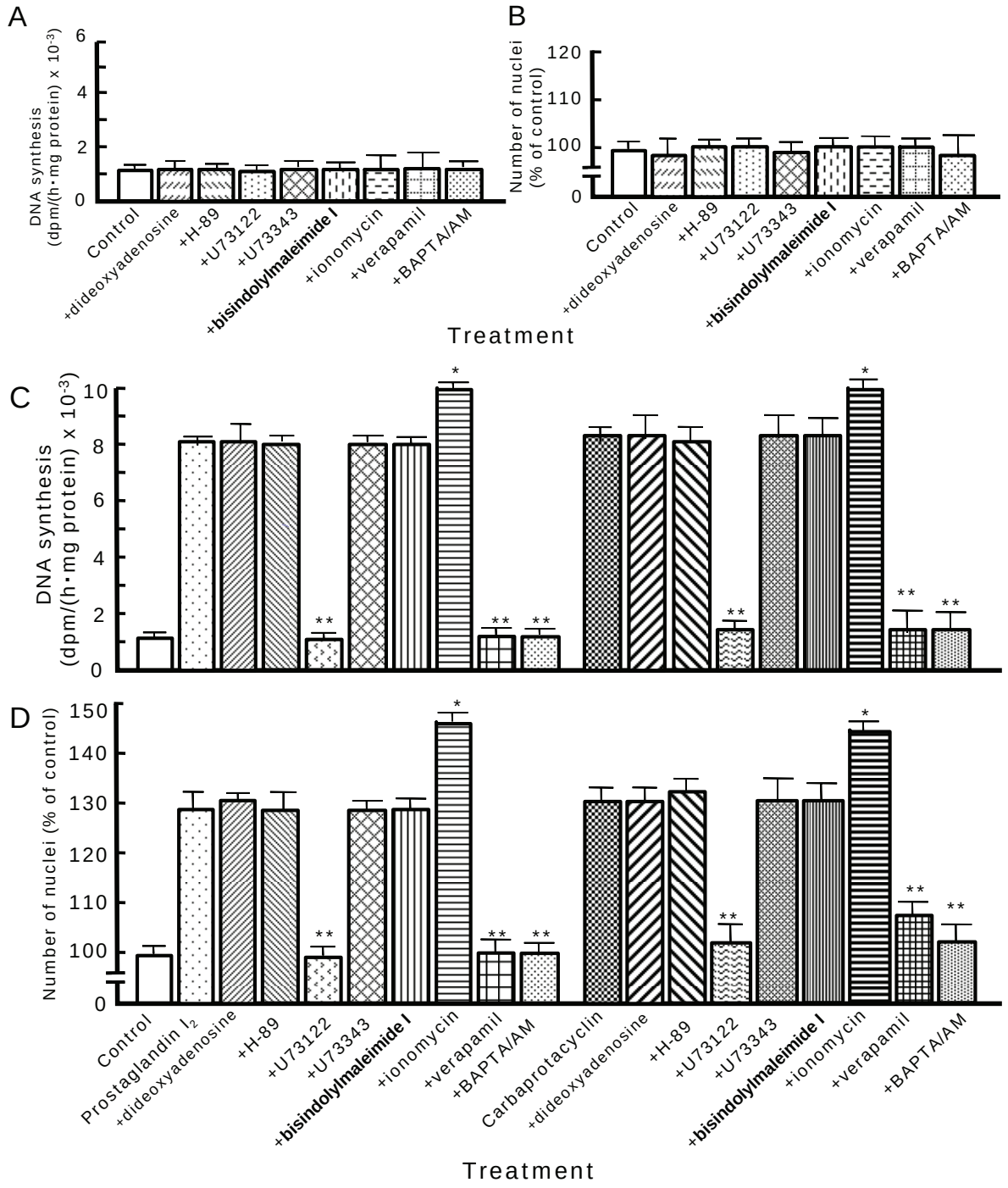

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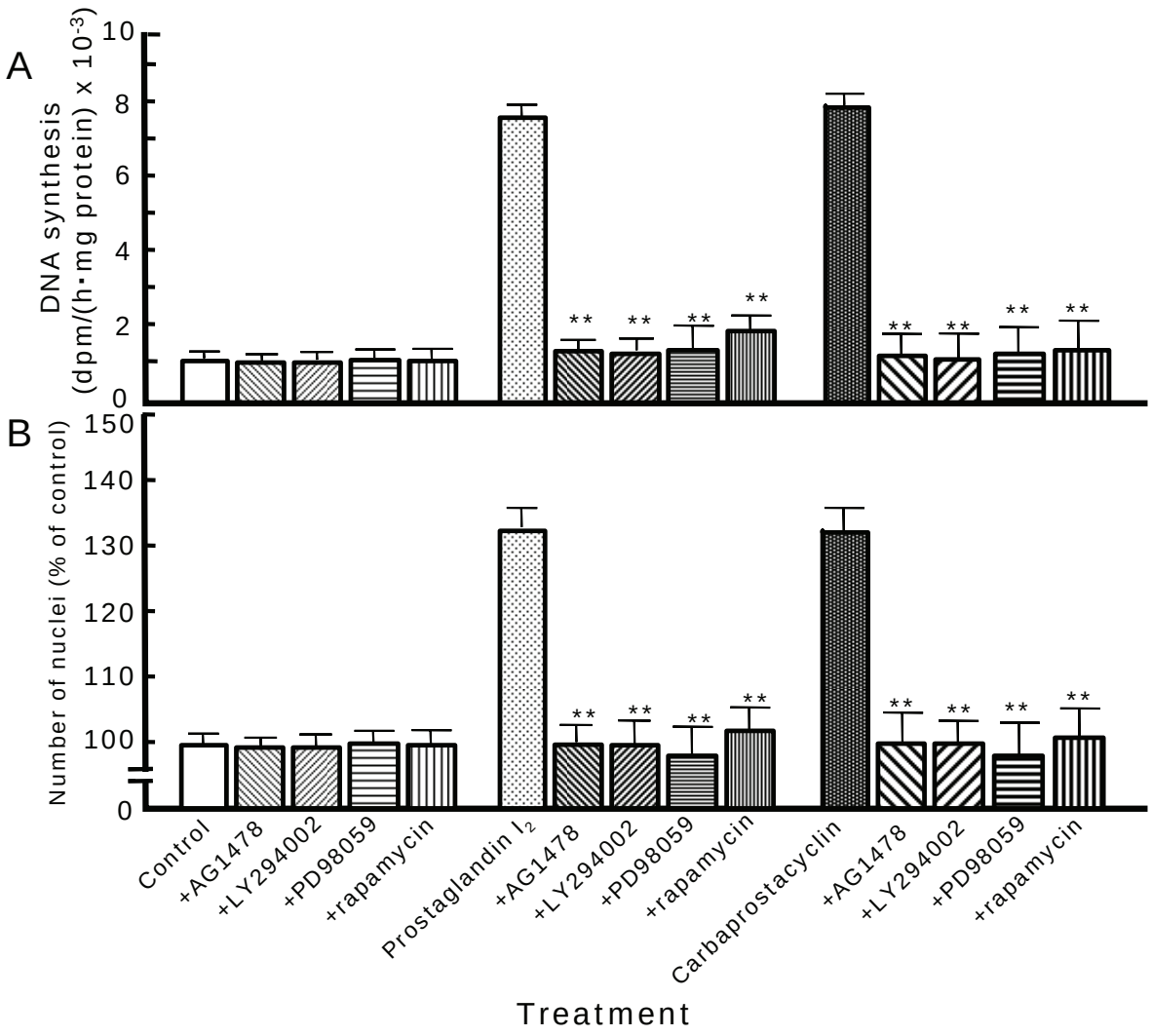

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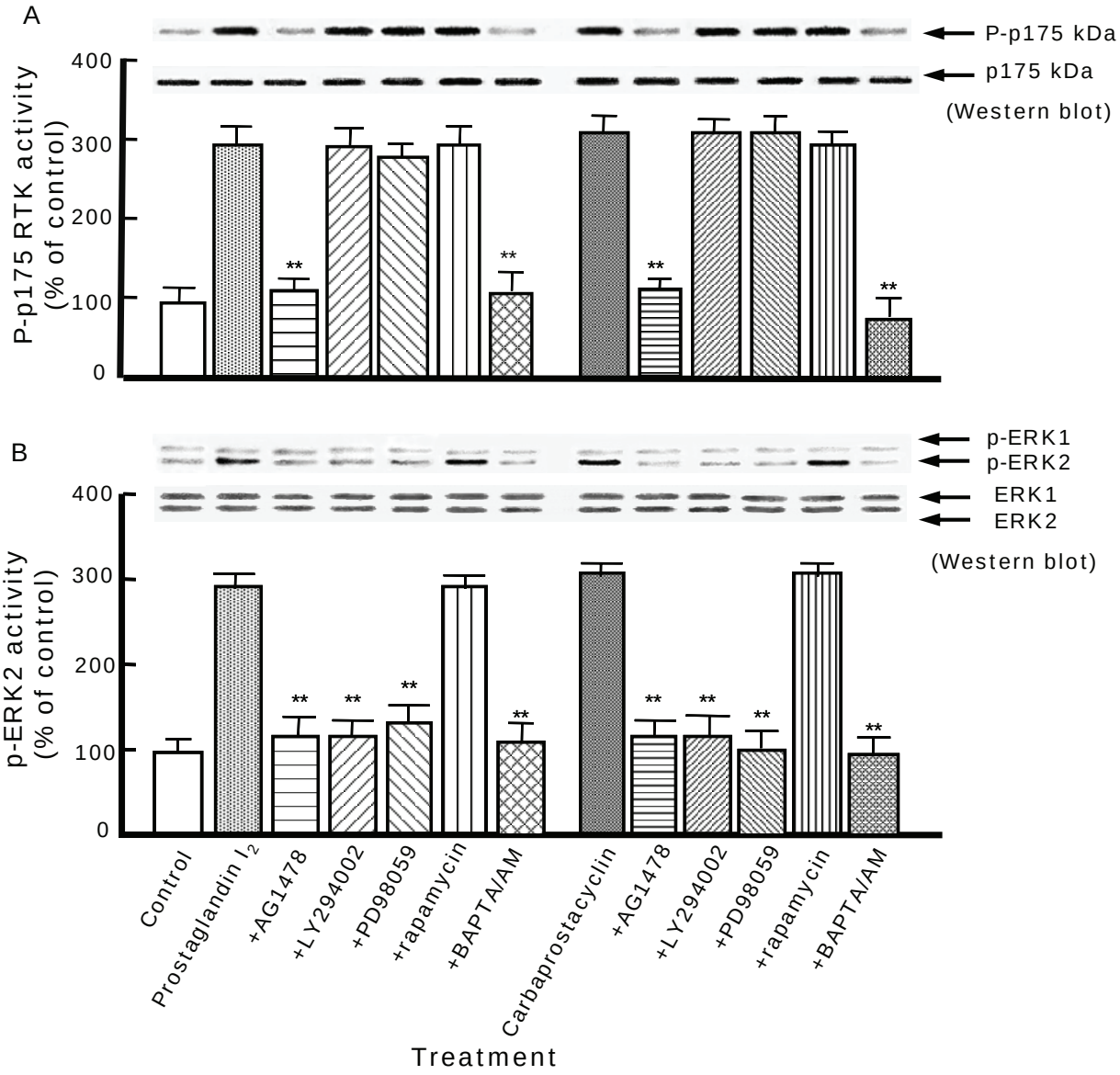

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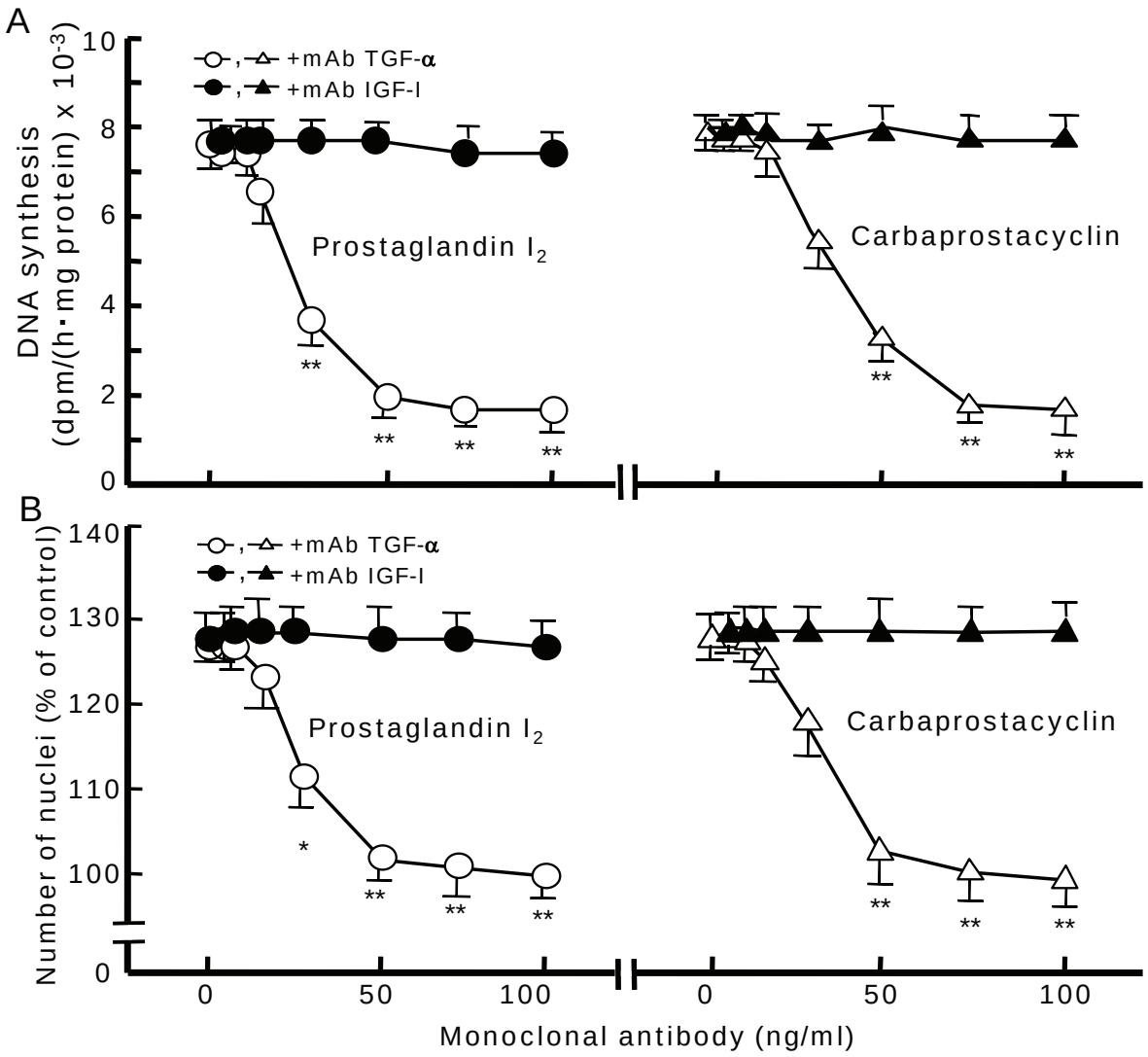

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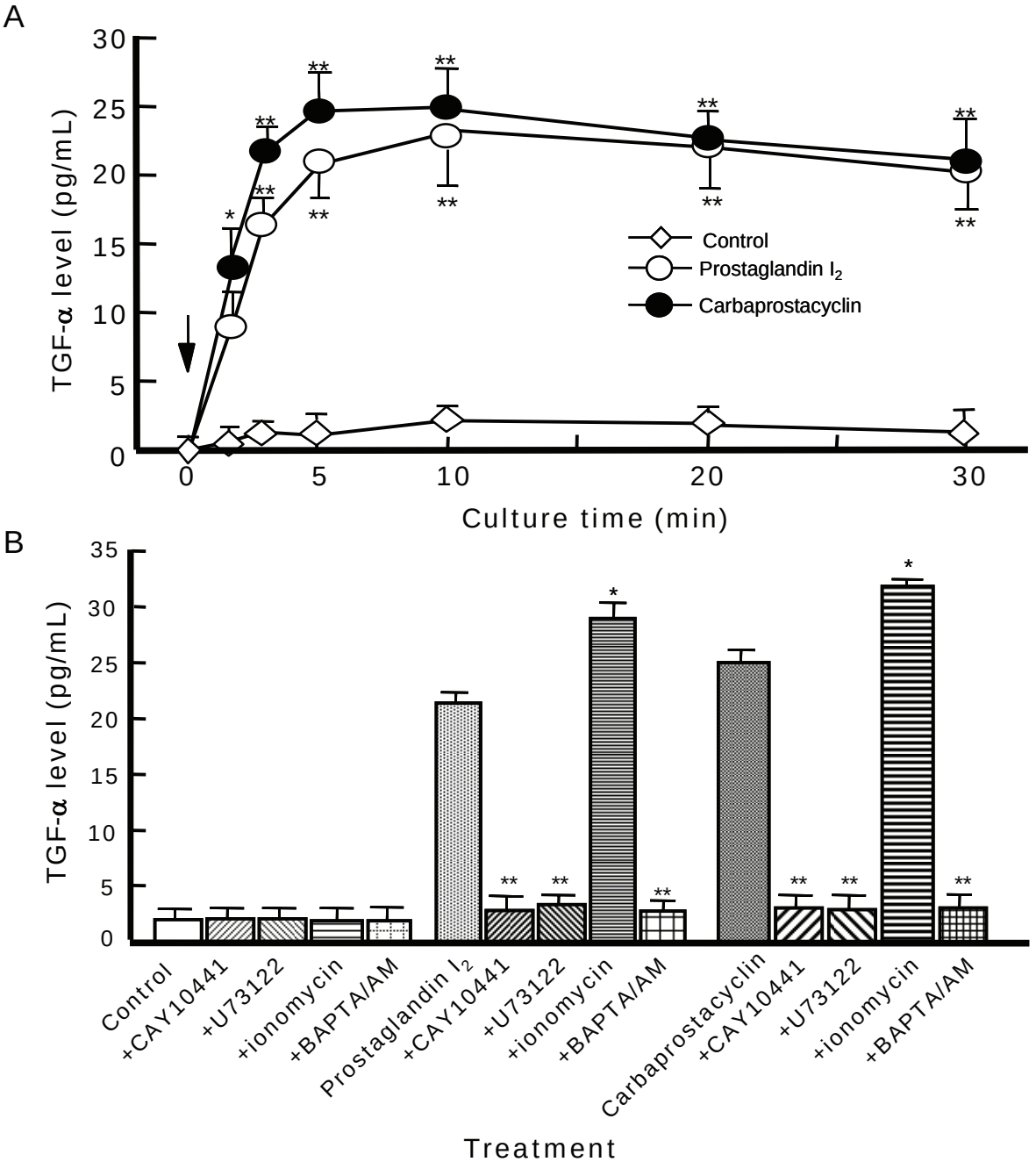

Fig.8 M. Kimura et al.