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骨芽細胞様細胞株（MC3T3-E1）の石灰化に対するプロスタグランジンE_2長期投与の効果

北海道大学大学院歯学研究科歯学臨床系専攻

（歯科矯正学講座）

かじい　たかし

梶井　貴史

指導

中村　進治　教授（北海道大学歯学部歯科矯正学講座）

松本　章　　教授（北海道大学歯学部歯科薬理学講座）

Long-term effects of prostaglandin E_2 on the mineralization of a clonal osteoblastic cell line (MC3T3-E1)

Takashi Kajii[a,*], Kuniaki Suzuki[b], Masatake Yoshikawa[a], Tohru Imai[a], Akira Matsumoto[b] and Shinji Nakamura[a]

[a]Department of Orthodontics, School of Dentistry, Hokkaido University, Kita 13, Nishi 7, Kita-ku, Sapporo 060-0813, Japan

[b]Department of Dental Pharmacology, School of Dentistry, Hokkaido University, Kita 13, Nishi 7, Kita-ku, Sapporo 060-0813, Japan

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*To whom correspondence should be addressed: Takashi Kajii: Department of Orthodontics, School of Dentistry, Hokkaido University, Kita 13, Nishi 7, Kita-ku, Sapporo 060-0813, Japan

Fax number: +81-11-706-4248 or +81-11-706-4917 (Japan)

E-mail address: kajii@den.hokudai.ac.jp

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Abbreviations: ALP, alkaline phosphatase, α-MEM, α-minimum essential medium, MES, 2-(N-morpholino) ethanesulfonic acid, PG, prostaglandin, *p*NPP, *p*-nitrophenyl phosphate, SDS, sodium dodecyl sulphate, TX, thromboxane.

Summary

Prostaglandin (PG) E_2 is thought to be a mediator of the effect of mechanical stress on bone formation; however the effects of PGE_2 on osteoblasts have not yet been fully described. We investigated the effects of the continuous application of PGE_2, and indomethacin, an inhibitor of prostaglandin G/H synthase (cyclooxygenase), on the proliferation, differentiation, and mineralization stage of a clonal osteoblastic cell line, MC3T3-E1. The cells were cultured in media with either a high (1 μg/ml) or a low (1 ng/ml) concentration of PGE_2, with indomethacin (1 μg/ml), and, as controls, with neither agent present. The effects of the applied PGE_2 and indomethacin were assessed quantitatively. Indomethacin and a high concentration of PGE_2 increased the total protein, compared to the control and low PGE_2 cultures. Seven days after confluence, alkaline phosphatase (ALP) activity within the cells and extracellular matrices increased. The increase of the activity was highest with indomethacin present and lowest with a high concentration of PGE_2 present. ALP activity in the medium also increased, but only 21 days after confluence. The effects of the agents were similar to those on the activity within the cells and matrices. The accumulation of calcium, inorganic phosphate, and hydroxyproline was highest with indomethacin present. PGE_2 production was at its maximum when MC3T3-E1 cells were at confluence, and indomethacin inhibited the production. We also measured the specific [^{3}H]PGE_2 binding to the microsomal fraction of the

cell to examine the expression of PGE_2 receptor. The amount of $[^3H]PGE_2$ binding per mg of protein was the highest at confluence, then decreased, and again increased in the mineralizing stage. These results suggest that indomethacin increases the ALP activity and the accumulation of mineralized tissue in MC3T3-E1 cells, presumably by inhibiting the production of PGE_2. PGE_2 may signal suppression of mineralization as early as confluence.

1. Introduction

Several lines of evidence suggest that prostaglandins (PGs) may be the or one of the messengers controlling bone deposition and resorption during orthodontic tooth movement. For example, local injection of PGE_1 upon orthodontic patients accelerates tooth movement (Yamasaki et al., 1984). Mechanical stress up- or down-regulates the production of PGE_2 by osteoblasts and fibroblasts (Binderman et al., 1988; Ngan et al., 1990; Klein-Nulend et al., 1995; Smalt et al., 1997). Endogenous PGE_2 production induced by the mechanical stress may also be related to the proliferation and differentiation of osteoblasts and fibroblasts (Ozawa et al., 1990; Jones et al., 1991; Yousefian et al., 1995).

The effects of PGs on bone formation have been studied in vivo (Norrdin et al., 1990; Jee and Ma, 1997). Tang et al. reported that exogenous PGE_2 increased colony formation in cultures of cells isolated from neonatal rat calvariae (1996). Exogenous PGE_2 also affects osteoblasts (Raisz, 1995; Frost et al., 1997), but the nature of this response is not clear. In most of

the experiments where osteoblasts were used, both PGE_2 application times and observation periods were short and looked at cells in culture only after confluence and not at the stages of proliferation, differentiation, and mineralization.

We have previously studied the long-term effects of PGE_2 on a clonal osteoblastic cell line, MC3T3-E1 cells. The MC3T3-E1 cell line is a good model for the study of the role of PGE_2 in mineralization as alkaline phosphatase (ALP) activity increases after confluence and calcified bone-like tissue is formed (Kodama et al., 1981; Sudo et al., 1983). PGE receptors have recently been cloned and they have been pharmacologically classified into four subtypes: EP1, EP2, EP3, and EP4 (Coleman et al., 1994). It is known that EP1, EP4, and/or EP2 mRNAs are expressed at confluence in MC3T3-E1 cells (Kasugai et al., 1995; Suda et al., 1996).

In the present study, we cultured MC3T3-E1 cells until mineralization with different concentrations of PGE_2, with indomethacin, a prostaglandin G/H synthase (cyclooxygenase) inhibitor and, as controls, with neither agent present. The effects of PGE_2 on MC3T3-E1 cells were evaluated for changes in ALP activity and the production and accumulation of protein, collagen, calcium and inorganic phosphate into the cells and extracellular matrices. We also examined the changes in the amount of endogenous PGE_2 and PGE_2 receptors throughout the cell culture.

2. Materials and methods

2.1. Cell culture

A mouse clonal osteoblastic cell line, MC3T3-E1 cells, was donated by Dr. Kodama (Ohu University) and Dr. Kuboki (Hokkaido University). The MC3T3-E1 cells were harvested via trypsinization and resuspended at 5×10^4 cells/ml. The cells (0.5 ml of resuspension) were seeded into each well of a 24-well tissue culture plate and grown in an α-minimum essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum in a 5% CO_2 - 95% air atmosphere at 37°C. After 1 day, they were exposed to new media 1) with neither PGE_2 nor indomethacin present (control), 2) with 1 ng/ml concentration of PGE_2 present (low PGE_2), 3) with 1 μg/ml concentration of PGE_2 present (high PGE_2), and 4) with 1 μg/ml indomethacin present (indomethacin). Each medium was changed every 2 or 3 days and the concentrations of the agents were maintained throughout the culture. The cells were assayed at various times as indicated in each Figure. The mineralization process of the cells and the effects of the agents were quantitatively analyzed by biochemical assays, and the representative data were obtained from three independent experiments. Since MC3T3-E1 cells are known to form multiple cell layers and to accumulate mineralized extracellular matrices (Sudo et al., 1983), we did not separate the cells from the extracellular matrices after removal of the culture medium. Thereby, we used the term "cells and matrices" in this paper, referring to cells and extracellular matrices.

2.2. Endogenous prostanoids synthesis

For measuring the endogenous prostanoids production, the culture medium with neither PGE_2 nor indomethacin present and the one with 1 μg/ml indomethacin present were recovered from each well. PGE_2 and 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2 (prostacyclin), were measured with the use of enzyme immunoassay kits (PerSeptive Biosystems, Framingham, MA, U.S.A.), and $PGF_{2\alpha}$ and thromboxane (TX) B_2 were measured with the use of other enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI, U.S.A.), according to the protocols provided.

2.3. Alkaline phosphatase (ALP) activity

The ALP activity within the cells and matrices, and that in the medium were measured by an assay (Suzuki et al., 1994; Yoshimura et al., 1996) based on the method of Bessey et al. (1946) with extensive modification to measure the activity attached to the cells and matrices (unpublished results). At various times the medium was removed from each well, and the cells and matrices attached to the well were washed three times with 0.25 M sucrose. After adding 0.9 ml of a reaction mixture containing 50 μmol of carbonate buffer at pH 9.22, 25 μmol of sucrose and 1 μmol of $MgCl_2$, the plate was preincubated at 37°C in a bath. The enzyme reaction was started by an addition of 0.1 ml of 20 mM *p*-nitrophenyl phosphate (*p*NPP). After 20 min, 0.5 ml of the reaction mixture was extracted and

put into 1.5 ml of 0.6 N NaOH solution to develop color. The hydrolysis activity of *p*NPP was measured colorimetrically at 420 nm. For measuring the ALP activity in the medium, 0.15 ml of the medium was recovered from each well. After adding 0.25 ml of a solution containing 12.5 μmol of sucrose, 25 μmol of carbonate buffer at pH 10.35, and 0.5 μmol of $MgCl_2$ with or without 0.75 μmol of levamisole, an ALP inhibitor, 0.4 ml of the mixture was preincubated at 37°C. The reaction was started by an addition of 0.1 ml of 10 mM *p*NPP and was terminated by an addition of 1.5 ml of 0.6 N NaOH after 30 min. The absorbance was read at 420 nm. For measuring the ALP activity, the value obtained from the medium with levamisole was subtracted from the value without levamisole.

2.4. Protein concentration

After measuring the ALP activity in the wells as described above, the wells were washed twice with water, and the cells and matrices were solubilized by an addition of 10% sodium dodecyl sulphate (SDS). The protein concentration was evaluated by the use of DC Protein Assay (Bio Rad, Hercules, CA, U.S.A.). The procedure uses bovine serum albumin as a standard, and is not interfered by 10% SDS.

2.5. Measurement of hydroxyproline

For measuring the amount of collagen produced by the cells and

accumulated in the cell matrices, the amount of hydroxyproline was measured by the method of Woessner (1961). At first, the cells and matrices were recovered from each well after washing three times with water, and hydrolyzed with 6 N HCl at 110°C for 24 hours; then HCl was removed with a rotary evaporator. The resultant hydrolysate was dissolved in water and used as a sample. One ml of 0.05 M chloramine T solution was added to 0.05 ml of the sample and the mixture was left for 20 min at room temperature. One ml of 3.15 M perchloric acid was further added and the mixture was left for 5 min at room temperature. Finally, 1 ml of 20% (w/v) *p*-dimethylaminobenzaldehyde was added and the mixture was incubated for 20 min at 60°C. After cooling, the absorbance was read at 555 nm.

2.6. Measurement of calcium and inorganic phosphate

For evaluation of the extent of mineralization in the extracellular matrices, the accumulation of calcium and inorganic phosphate was measured. First, the hydrolysate obtained for the measurement of hydroxyproline was transferred into a porcelain crucible and burnt to ashes for 18 hours at 750°C (Corradino et al., 1971). For measuring the amount of calcium, the ashes were dissolved in 5% (v/v) HCl containing 1% (w/v) $LaCl_3$ (Corradino et al., 1971) to avoid interference of phosphate, and assayed with a Varian AA-1475 atomic absorption spectrophotometer. For measuring the amount of inorganic phosphate, the ashes were dissolved in water and 300 μl of the sample was taken,

which was assayed as described by Chifflet et al. (1988).

2.7. PGE_2 binding assay

MC3T3-E1 cells were cultured in a usual α-MEM with neither PGE_2 nor indomethacin present in 100 mm plastic dishes. At appropriate times during the 0-35 days after confluence, the cells were harvested, ultrasonicated twice for 20 seconds at 20-second interval, and then homogenized in 0.25 M sucrose. The homogenate was separated by centrifugation according to the method of Hogeboom (1955) with some modification, and the resultant microsomal fraction was used for the [^{3}H]PGE_2 binding assay. Twenty to 60 μg of the microsomal fraction was incubated with 2 nmol of [^{3}H]PGE_2 in 0.1 ml of the reaction mixture containing 2 μmol of 2-(N-morpholino) ethanesulfonic acid (MES) at pH 6.0, 1 μmol of $MgCl_2$ and 0.1 μmol of EDTA for 1 hour at 30°C (Sugimoto et al., 1993). In the preliminary experiments, the specific [^{3}H]PGE_2 binding was saturated under the above experimental condition. After incubation, the mixture was immediately centrifuged at 20,000 $\times g$ for 30 min, and the radioactivity in the supernatant was measured with a liquid scintillation counter. For obtaining the counts in the pellet, the counts in the supernatant were subtracted from those of added [^{3}H]PGE_2. The counts in the pellet obtained in the presence of 100-fold excess of unlabeled PGE_2 were considered as the nonspecific binding. The specific binding was calculated by subtracting the nonspecific binding from the counts in the pellet without unlabeled PGE_2.

2.8. Chemicals

PGE_2 was purchased from Sigma (St. Louis, MO, U.S.A.); indomethacin from Wako Ltd, (Osaka, Japan); [^{3}H]PGE_2 from Amersham (Buckinghamshire, England); and other chemicals were all in analytical grades.

2.9. Statistical analysis

Data obtained were subjected to the *F*-test, and then Student's *t*-test or Welch's *t*-test.

3. Results

3.1. Characterization of MC3T3-E1 Cells

MC3T3-E1 cells rapidly grew after plating and formed fibroblast-like shapes until they reached the confluence. After confluence, they changed to polygonal shapes. They continued to grow thereafter and formed multilayered structures. At about day 14 after confluence, colonies with clusters of characteristic spherical cells and small mineralized opaque nodules appeared. These colonies steadily increased in number and size, and about 30 days after confluence, the nodules also multiplied.

3.2. Production of prostanoids by MC3T3-E1 cells

We measured the productions of PGE_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$ and TXB_2 by MC3T3-E1 cells during the cell culture. 6-keto $PGF_{1\alpha}$ is the stable metabolite of PGI_2 (prostacyclin). As shown in Fig. 1, PGE_2 was the major prostanoid produced into the control medium (with neither PGE_2 nor indomethacin). The PGE_2 synthesis was the highest at confluence, and then it decreased. However, in the medium with 1 μg/ml indomethacin, either PGE_2 or any other prostanoid was hardly detectable throughout the experimental period (data not shown).

3.3. Effects of indomethacin and PGE_2 on the protein content in the MC3T3-E1 cells and the extracellular matrices

The protein content, in accordance with the cell growth and the accumulation of extracellular matrices produced by cells, increased during the experimental period. In particular, indomethacin (1 μg/ml) or a high concentration (1 μg/ml) of PGE_2 increased the protein content more markedly than the control (without addition) and low (1 ng/ml) PGE_2 (Fig. 2).

3.4. ALP activity in the cells and matrices

The ALP activity in the MC3T3-E1 cells increased from day 14 after confluence in all the culture conditions (Fig. 3A). Among them, the

activity increased most significantly under the condition with the indomethacin treatment from 7 to 28 days after confluence. During the period of from day 14 to 28, the increase was most significant with indomethacin present, which was followed by the control, and with low PGE_2 present, and the least increase was with high PGE_2 present.

3.5. ALP activity in the culture medium

The ALP activity released from the cells into the medium also increased in all the culture conditions (Fig. 3B), and the increase was about 10 days behind that in the cells and matrices. Similarly to the increase in the cells and matrices, the most significant increase of activity was with indomethacin present, which was followed by the control, and with low PGE_2 present, and the least increase was with high PGE_2 present.

3.6. Accumulation of hydroxyproline in the extracellular matrices

To evaluate the synthesis and accumulation of collagen by the MC3T3-E1 cells, we measured the hydroxyproline content in the cells and matrices after mineralized opaque nodules had appeared (Fig. 4A). The amount of hydroxyproline increased from day 21 to 35 in all the culture conditions, and most significantly increased in the condition with indomethacin present.

3.7. Accumulation of calcium and inorganic phosphate

The amounts of calcium and inorganic phosphate accumulated in the cells and matrices hardly changed till day 14 after confluence, and then increased from day 21 to 35 in all the culture conditions (Fig. 4B, C). Among all the treatment agents, indomethacin increased both accumulations most significantly.

3.8. Chronological changes in PGE_2 binding to the membrane fraction of MC3T3-E1 cells

The effects of PGE_2 on the cells should be exerted by binding to its receptors; therefore, to study the expression of PGE_2 receptors, we measured the binding capacity of $[^3H]PGE_2$ to the membrane fraction of MC3T3-E1 cells. The specific binding of $[^3H]PGE_2$ to the fraction was saturated at 20 μM (data not shown). As shown in Fig. 5, $[^3H]PGE_2$ binding per mg of protein was highest at confluence, and gradually declined until day 18, after which it increased again.

Discussion

Numerous reports set out the effects of PGE_2 on ALP activity and mineralization of osteoblasts (Raisz et al., 1993b; Kawaguchi et al., 1995); however, they have not yet been fully elucidated, and experimental findings disagree. Reasons for such discrepancy may lie in differences in the cells and experimental conditions. Few reports have described the

effects of PGE_2 on the mineralization stage of osteoblasts in vitro; and in some of the experiments, the osteoblastic cells may not have formed mineralized tissue. In most of the previous studies, PGE_2 was added to the culture medium to stimulate the cells for only short periods. In the present study, we examined the effects of PGE_2 on osteoblastic cells for a longer period.

The cells we used were MC3T3-E1, which undergo three stages of development: proliferation, differentiation, and mineralization (Quarles et al., 1992). The first stage is the initial phase of development (until about 5 days after confluence), in which the cells actively replicate; in the next stage (about 5 days after confluence), the cells express ALP activity and the cell growth slows down; in the final stage (at approximately day 20), calcification of matrix occurs. The cells cultured in the control medium (with neither PGE_2 nor indomethacin present) underwent the three stages of the regular development process.

To determine the effects of PGE_2 and indomethacin on the proliferation, differentiation, and mineralization of the cells, we cultured the cells in the following 4 types of the media: 1) usual α-MEM (control), 2) α-MEM containing 1 ng/ml PGE_2 (low PGE_2), 3) α-MEM containing 1 μg/ml PGE_2 (high PGE_2), and 4) α-MEM containing 1 μg/ml indomethacin (indomethacin). The agents were continuously applied throughout the cell culture.

PGE_2 was found to be the major prostanoid produced by the MC3T3-E1 cells; the cells produced 28.5 ± 5.2 ng/ml of PGE_2 in the control medium at confluence (Fig. 1). The PGE_2 production was, however, almost

completely inhibited by the addition of 1 μg/ml of indomethacin. This finding agreed with the previous reports (Yokota et al., 1986; Igarashi et al., 1994). As the total amount of PGE_2 in the culture medium should be the sum of applied and endogenously secreted PGE_2, we, therefore, assumed that the PGE_2 concentration would be highest in the culture medium with high PGE_2 present, which would be followed by that in the medium with low PGE_2 present and then that in the control and lastly by the one in the medium with indomethacin present. In the present study, as we did not add indomethacin to the cultures of control, low PGE_2, and high PGE_2, endogenous PGE_2 production should have continued during our experiments. Therefore, we did not differentiate the effects of applied PGE_2 from those of endogenously secreted PGE_2. Endogenous PGE_2 production may vary under the different culture conditions. Further experiments in which endogenous PGE_2 production is suppressed would be needed to clarify the respective effects of applied and endogenously secreted PGE_2 quantitatively.

We measured hydroxyproline, calcium, and inorganic phosphate to evaluate the extent of mineralization quantitatively, although microscopic observation and von Kossa staining are often used to evaluate the mineralization. We developed a method to assay the ALP activity in the cells directly in each well so that a loss of activity in the recovery of the cells could be prevented, as described in the Materials and methods. The ALP activity in the medium was also measured, which was important for evaluating all the ALP activity generated by the cells.

The addition of 1 μg/ml indomethacin increased the ALP activity in the

cells and matrices (Fig. 3A), and that in the culture medium (Fig. 3B); and it also increased the accumulation of hydroxyproline, calcium, and inorganic phosphate (Fig. 4). Since the degree of these activities and accumulations tended to be dependent on the PGE_2 concentration in the medium, we suggest that PGE_2 inhibits ALP activity and mineralization of MC3T3-E1 cells. These results are supported by the previous reports that PGE_2 inhibits the collagen synthesis of osteoblasts (Raisz et al., 1993a; Centrella et al., 1994; Fall et al., 1994; Harrison et al., 1994) and that PGE_2 inhibits mineralization of osteoblasts (Morita et al., 1989; Ogiso et al., 1992). However, Igarashi et al. (1994) reported that a continuous addition of indomethacin (1 μg/ml) did not affect the calcium content. The different finding may have arisen from the differences in the culture medium and the incubation period.

The present results also suggested that ALP was released to some extent from the cells into the medium (Fig. 3B). The ALP activity increased in the medium about 10 days after the increase in the cells and matrices, corresponding to the mineralization stage (Figs. 3 and 4). From these findings, we speculate that ALP matures while being attached to the cells, and that the matured ALP is necessary for the mineralization.

In the present study, 1 μg/ml indomethacin and 1 μg/ml PGE_2 both increased the protein content, which we think was caused by the cell growth and the accumulation of the extracellular matrices, compared to those in other conditions (Fig. 2). Our results indicate that 1 μg/ml indomethacin enhanced the collagen synthesis (Fig. 4A). According to Hakeda et al. (1985; 1986), an addition of more than 500 ng/ml PGE_2

increases DNA synthesis and suppresses ALP activity, and that less than 500 ng/ml PGE_2 decreases DNA synthesis and stimulates ALP activity in MC3T3-E1 cells for only a few days after confluence. Their findings support our hypothesis that 1 μg/ml PGE_2 enhances the cell replication.

The amount of bound PGE_2 per mg of protein was the highest at confluence and then declined. This suggests that the expression of PGE_2 receptors per cell reaches its maximum at confluence. It is reported that whereas collagen accumulation increases at about 10 days after confluence, the expression of procollagen mRNA (Quarles et al., 1992) and the amount of precursors of the cross-link (Kuboki et al., 1992) reach their maximums at confluence and then decrease in MC3T3-E1 cells. Furthermore, the PGE_2 production by MC3T3-E1 cells reached its maximum at confluence in the present study (Fig. 1), as reported previously by others (Farr et al., 1984; Igarashi et al., 1994; Sun et al., 1997). These results suggest that PGE_2 may signal suppression of mineralization already at confluence.

Pharmacologically, PGE receptors have been classified into four subtypes: EP1, EP2, EP3, and EP4. These subtypes have recently been cloned and characterized in detail (Coleman et al., 1994). Suda et al. (1996) and Kasugai et al. (1995) reported that MC3T3-E1 cells expressed EP1 and EP4 and/or EP2 mRNAs at confluence. Suda et al. (1996) further revealed that EP1 up-regulated DNA synthesis and down-regulated ALP activity whereas EP4 down-regulated DNA synthesis and up-regulated ALP activity in MC3T3-E1 cells at confluence, by using the agonists specific to PGE_2 receptor subtypes. Further, Hakeda et al. (1987)

reported that the biphasic effects of a high or a low PGE_2 concentration on DNA synthesis and ALP activity might be mediated by two different receptors. Along with these reports, the present results suggest that the decrease of ALP activity by 1 μg/ml PGE_2 may be mediated by EP1 receptors and that indomethacin inhibits the PGE_2 production and increases the ALP activity by reducing the signal from PGE_2.

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Figure Legends

Fig. 1. Changes in syntheses of prostanoids during culture of MC3T3-E1 cells. The culture medium with neither PGE_2 nor indomethacin (control) was recovered from each well at various days of culture. The amounts of PGE_2 (●), $PGF_{2\alpha}$ (O), 6-keto $PGF_{1\alpha}$ (■) and TXB_2 (□) were measured

by enzyme immunoassay kits according to the protocols provided. The data represent the mean ± SD of 4 wells (n=4). Most of the SD was included in the symbols. PGE_2 synthesis was significantly high at confluence, which was followed by that on day 4 after confluence. $^{**}p < 0.01$ (Student's *t*-test).

Fig. 2. Changes in the protein content of the MC3T3-E1 cells caused by indomethacin or exogenous PGE_2 addition. The cells were grown without any addition (control) (O), with 1 μg/ml indomethacin present (indomethacin) (●), 1 ng/ml PGE_2 present (low PGE_2) (■), and 1 μg/ml PGE_2 present (high PGE_2) (▲) throughout the culture period. The cells and the extracellular matrices were solubilized by 10% SDS and the protein content was evaluated by DC Protein Assay (Bio Rad). The data represent the mean ± SD of 6 wells (n=6). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control (Student's *t*-test or Welch's *t*-test).

Fig. 3. Changes in ALP activities in the cells and matrices (A) and in the culture medium (B), caused by indomethacin or exogenous PGE_2 addition. The cells were grown without any addition (control) (O), with 1 μg/ml indomethacin (indomethacin) (●), 1 ng/ml PGE_2 (low PGE_2) (■) and 1 μg/ml PGE_2 (high PGE_2) (▲) throughout the culture period. After the medium was removed from each well, the activity attached to the cells and matrices (A) was measured as described in Materials and methods. The activity in the medium (B) was also determined. The data represent the mean ± SD of 4 wells (n=4). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control

(Student's *t*-test or Welch's *t*-test).

Fig. 4. Hydroxyproline (A), calcium (B), and inorganic phosphate (C) accumulations during MC3T3-E1 development in the absence or presence of indomethacin or exogenous PGE_2. The cells were grown without any addition (control) (open columns), with 1 μg/ml indomethacin (indomethacin) (solid columns), 1 ng/ml PGE_2 (low PGE_2) (diagonally hatched columns) and 1 μg/ml PGE_2 (high PGE_2) (horizontally hatched columns) throughout the culture period. Hydroxyproline was measured by the method of Woessnor. For measuring calcium and phosphate, hydrolysate was burnt to ashes for 18 hours at 750°C. Then, the calcium content was assayed with atomic absorption spectrophotometry. Inorganic phosphate was measured by the method of Chifflet et al. The data represent the mean ± SD of 4 samples (n=4). $*p < 0.05$ and $**p < 0.01$ versus control (Student's *t*-test or Welch's *t*-test).

Fig. 5. Changes in PGE_2 binding to the microsomal fraction of MC3T3-E1 cells after different periods of culture. The microsomal fraction prepared by the method of Hogeboom with modification was used for the $[^3H]PGE_2$ binding assay. Twenty to 60 μg of the microsomal fraction was incubated with 2 nmol of $[^3H]PGE_2$ in 0.1 ml of the reaction mixture containing 2 μmol of MES at pH 6.0, 1 μmol of $MgCl_2$, and 0.1 μmol of EDTA for 1 hour at 30°C. After incubation, the mixture was centrifuged at 20,000 $\times g$ for 30 min, and the radioactivity was counted on a liquid scintillation counter. The counts obtained in the presence of 100-fold

excess of unlabeled PGE_2 were considered as the nonspecific binding. The data represent the mean ± SD of triplicate experiments.

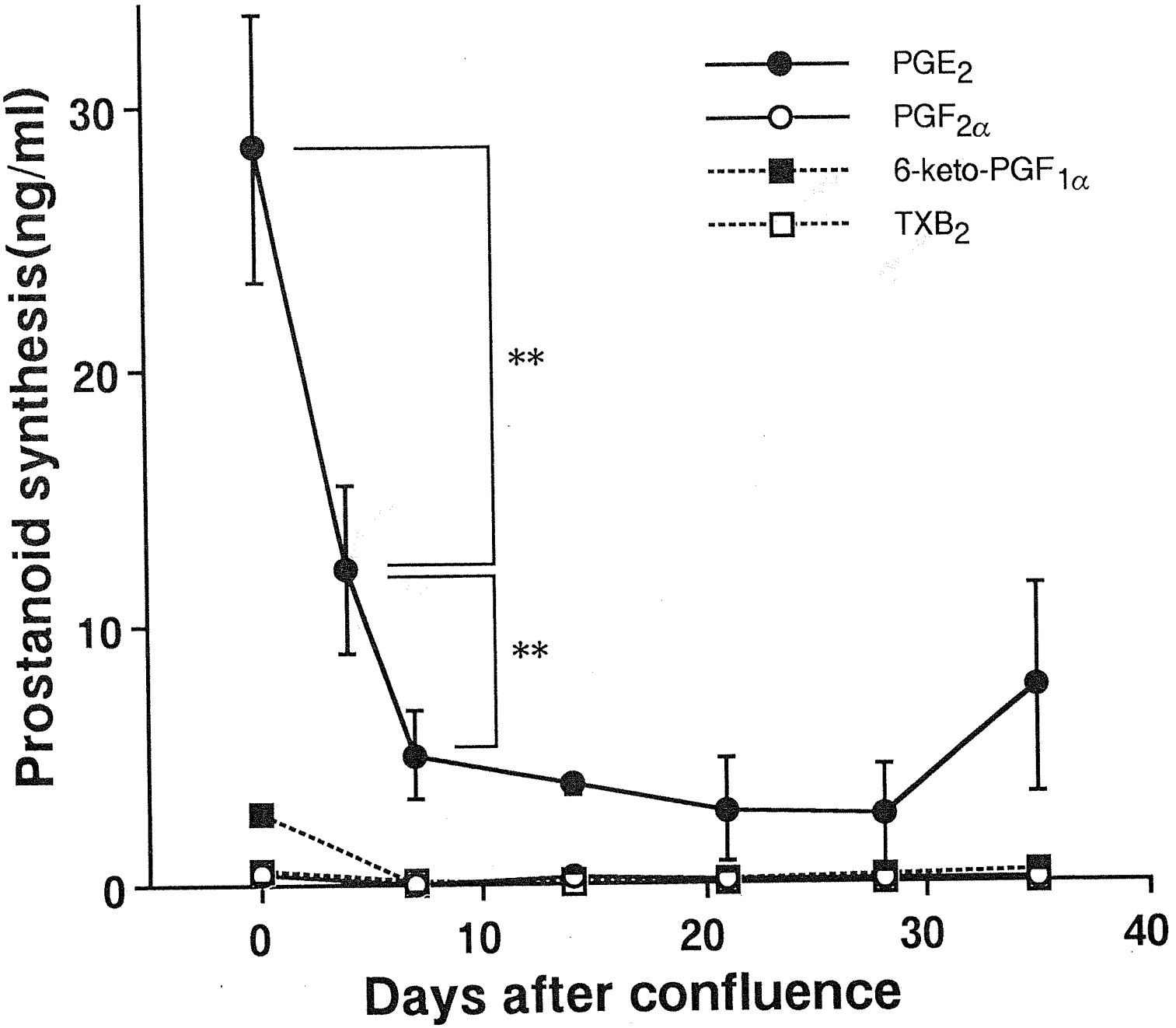
Prostanoid synthesis(ng/ml)
30
20
10
0
0
10
20
30
40
Days after confluence
PGE2
PGF2α
6-keto-PGF1α
TXB2
**
**

Fig. 1

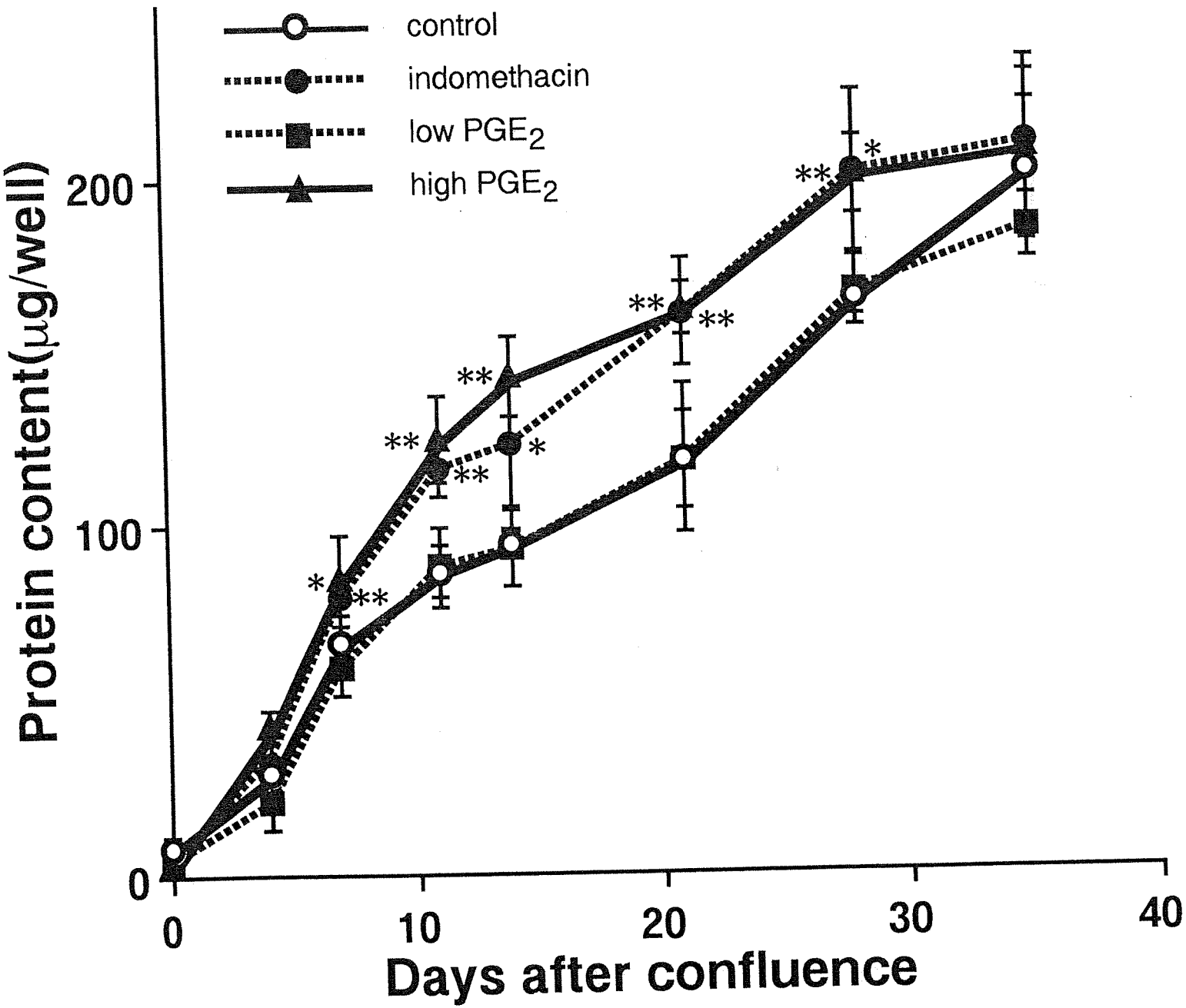
control
indomethacin
low PGE2
high PGE2
Protein content(μg/well)
200
100
0
0
10
20
30
40
Days after confluence

Fig. 2

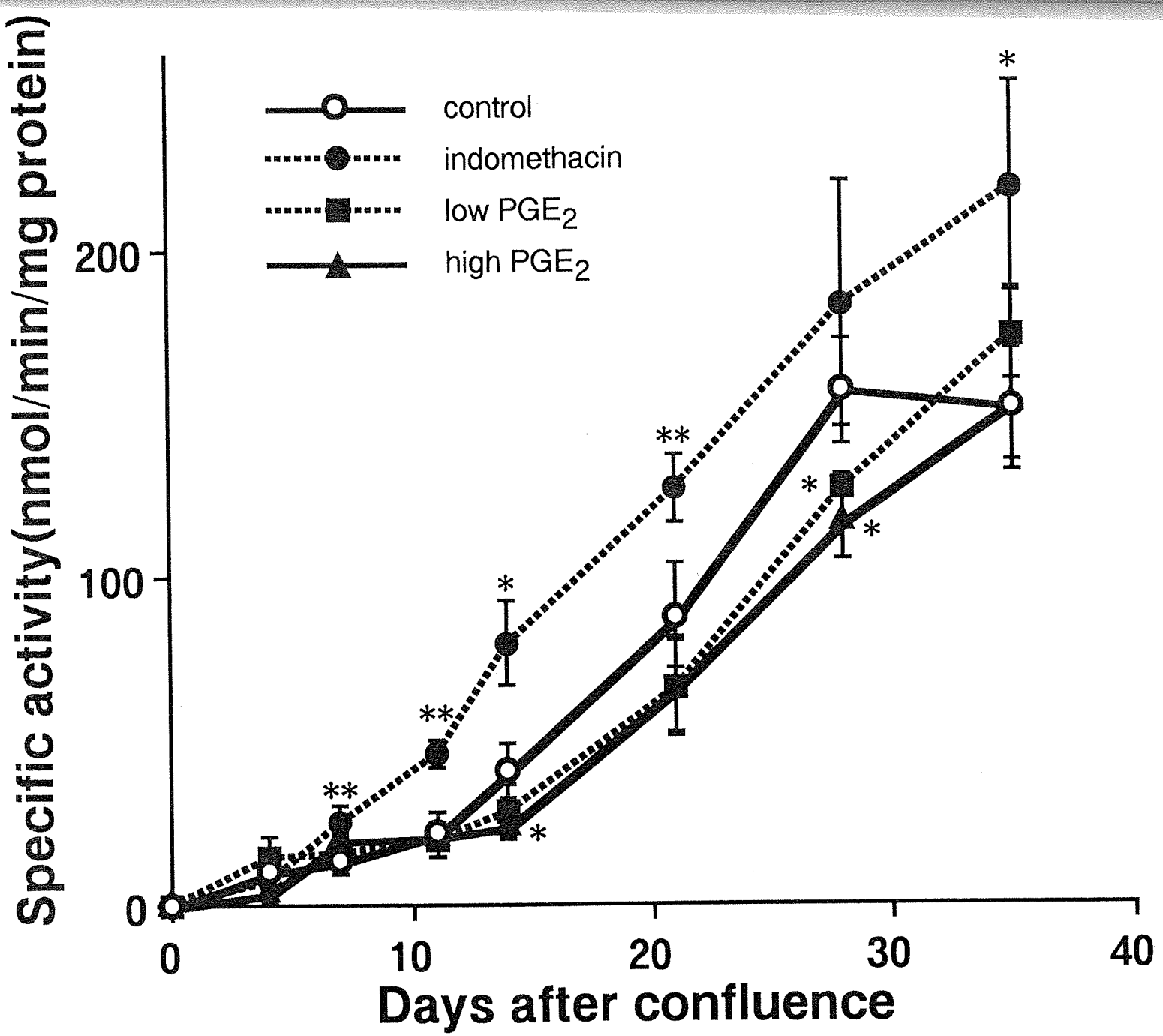
control
indomethacin
low PGE2
high PGE2
Specific activity(nmol/min/mg protein)
0
100
200
0
10
20
30
40
Days after confluence
*
**

Fig. 3A

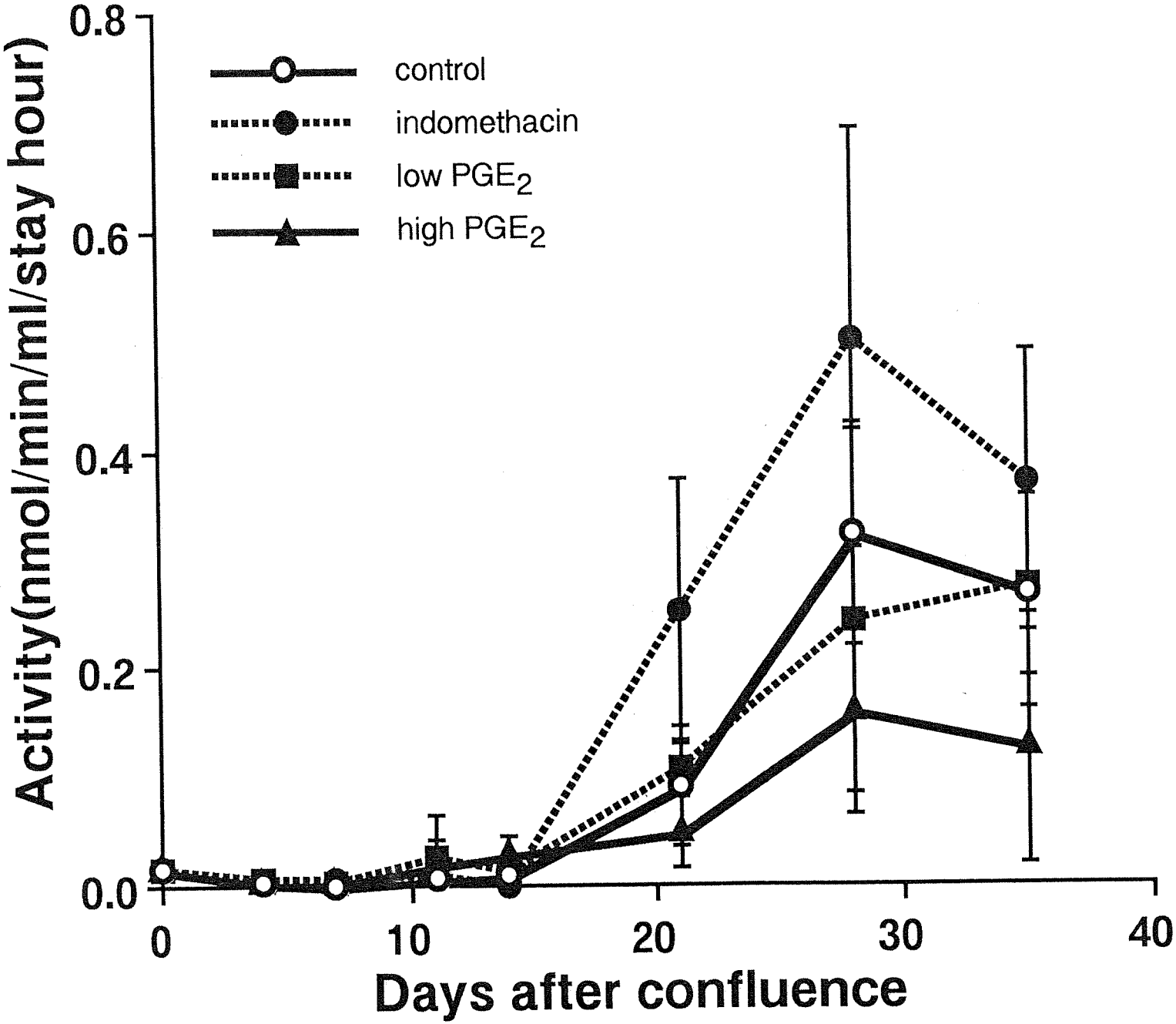
0.8
0.6
0.4
0.2
0.0
Activity(nmol/min/ml/stay hour)
control
indomethacin
low PGE2
high PGE2
0
10
20
30
40
Days after confluence

Fig. 3B

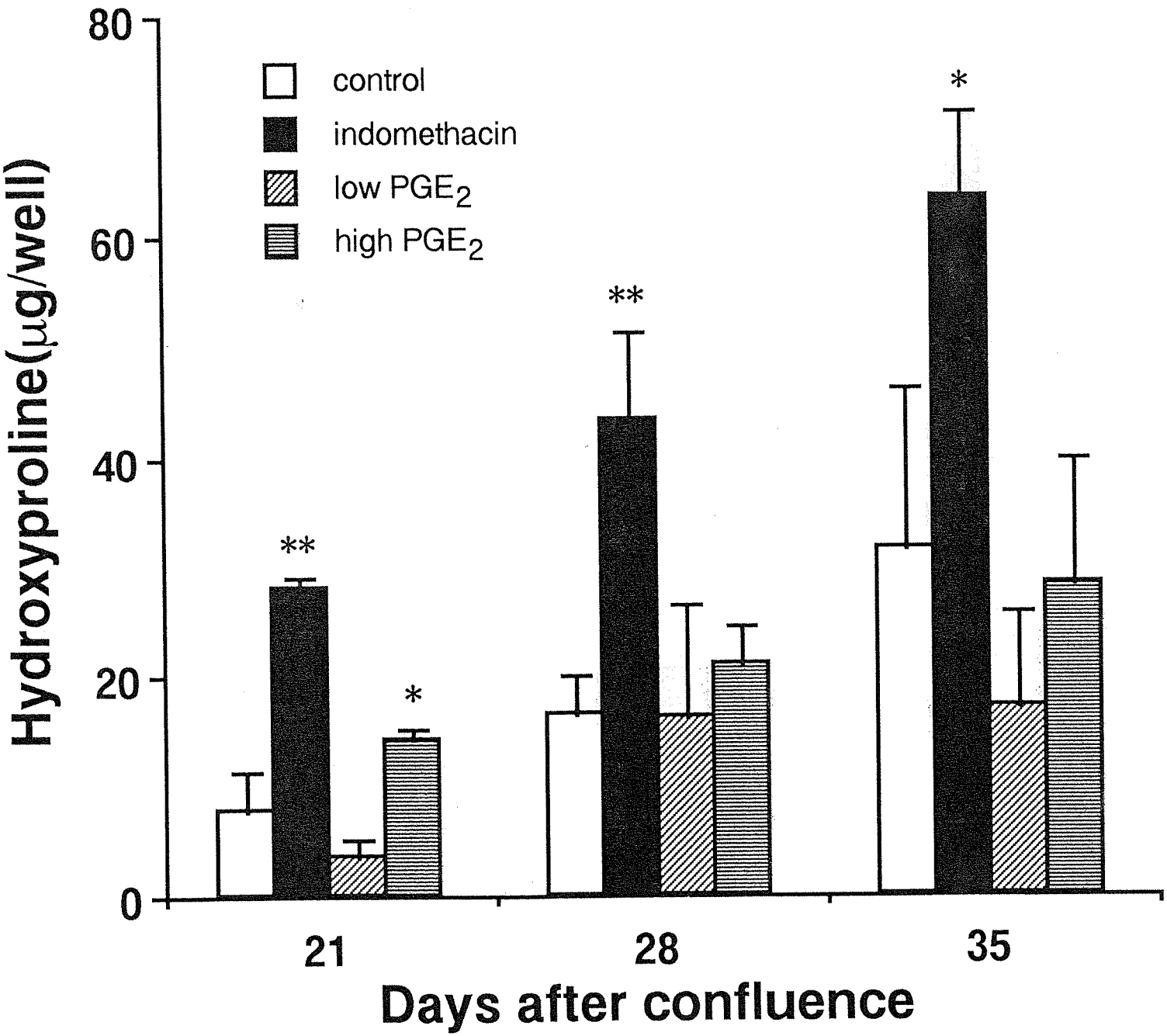
80
60
40
20
0
Hydroxyproline(μg/well)
control
indomethacin
low PGE2
high PGE2
**
*
**
*
21
28
35
Days after confluence

Fig. 4A

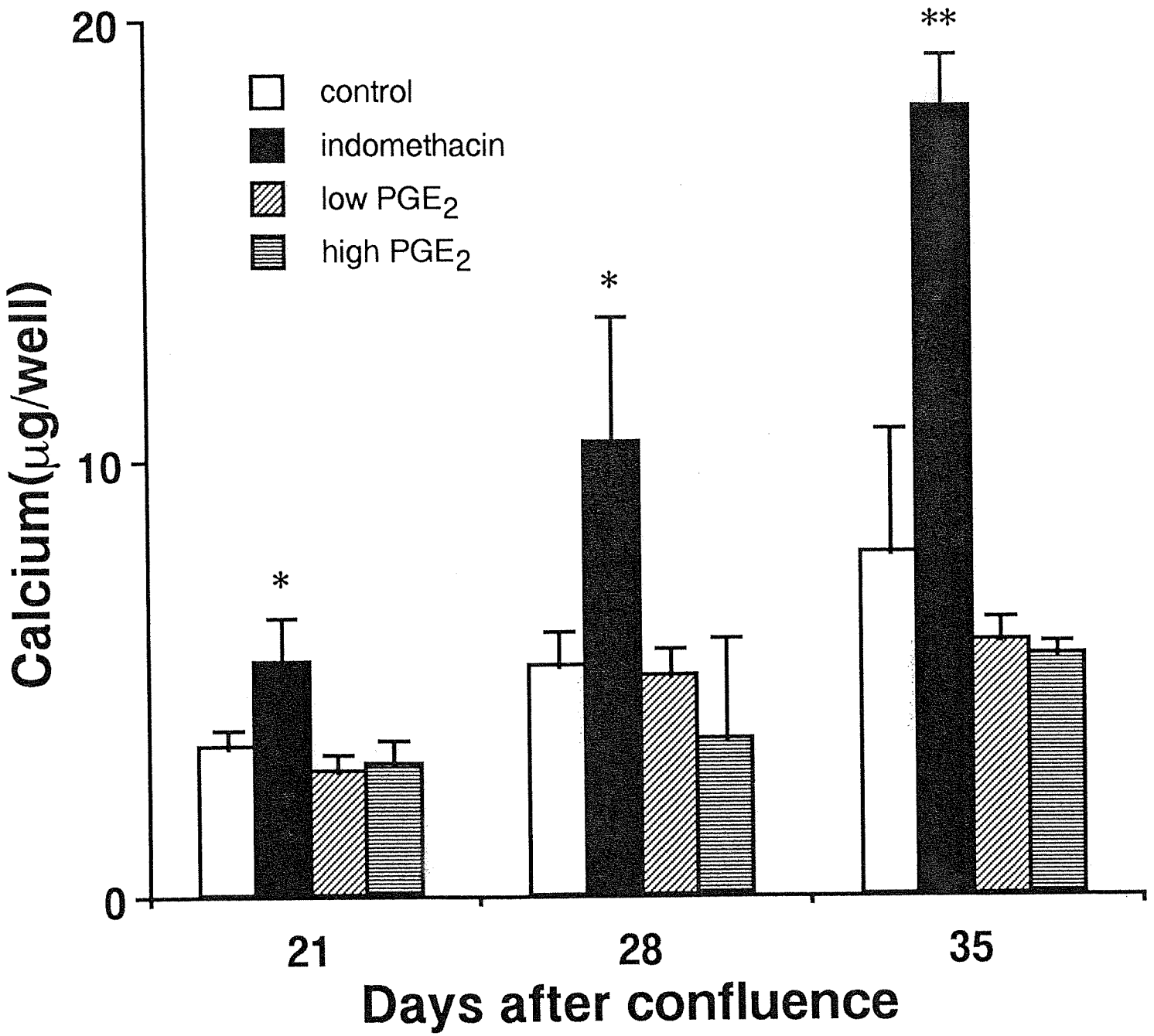
20
10
0
Calcium(μg/well)
control
indomethacin
low PGE2
high PGE2
*
*
**
21
28
35
Days after confluence

Fig. 4B

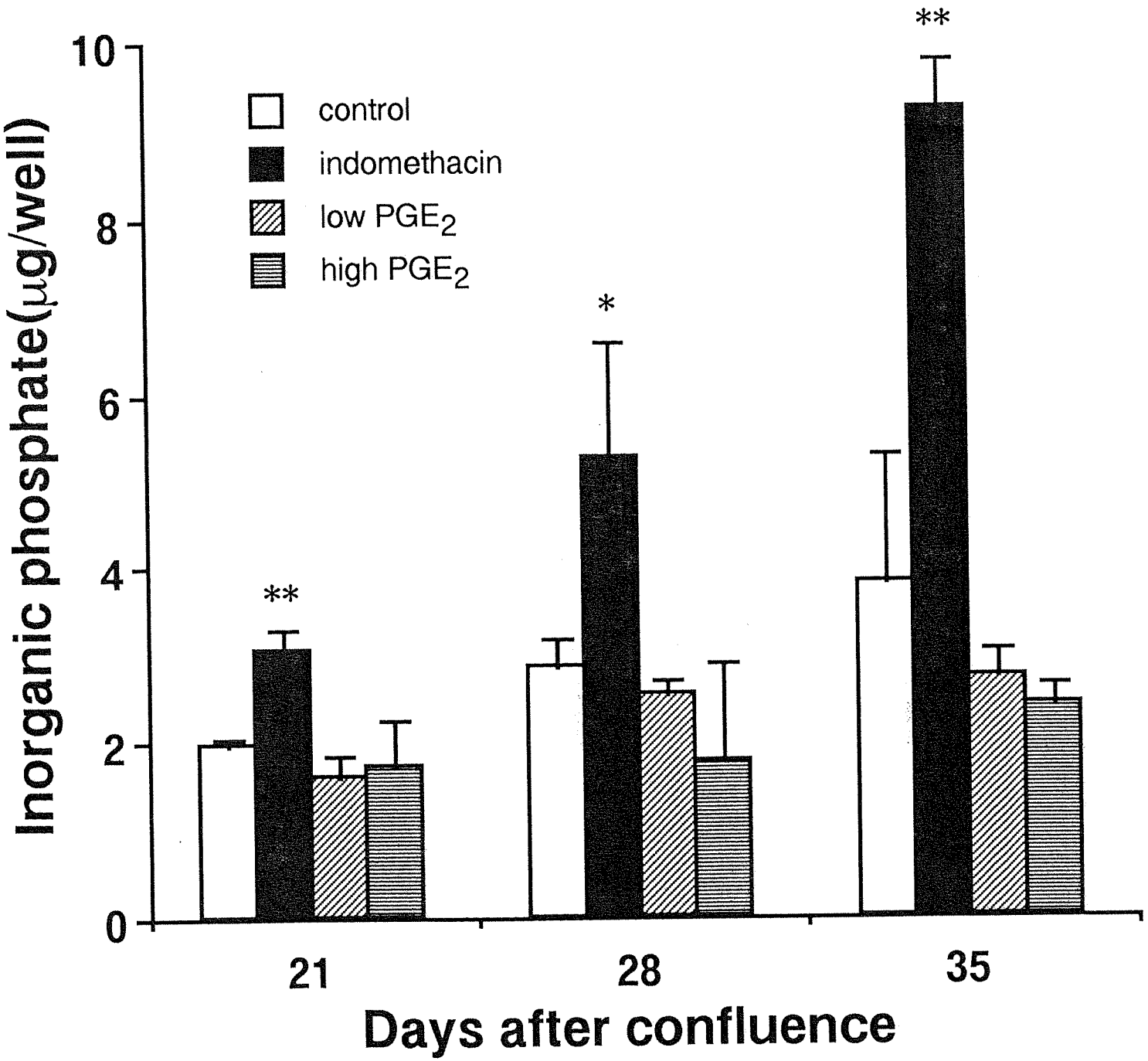
Inorganic phosphate(μg/well)
10
8
6
4
2
0
control
indomethacin
low PGE2
high PGE2
**
*
**
21
28
35
Days after confluence

Fig. 4C

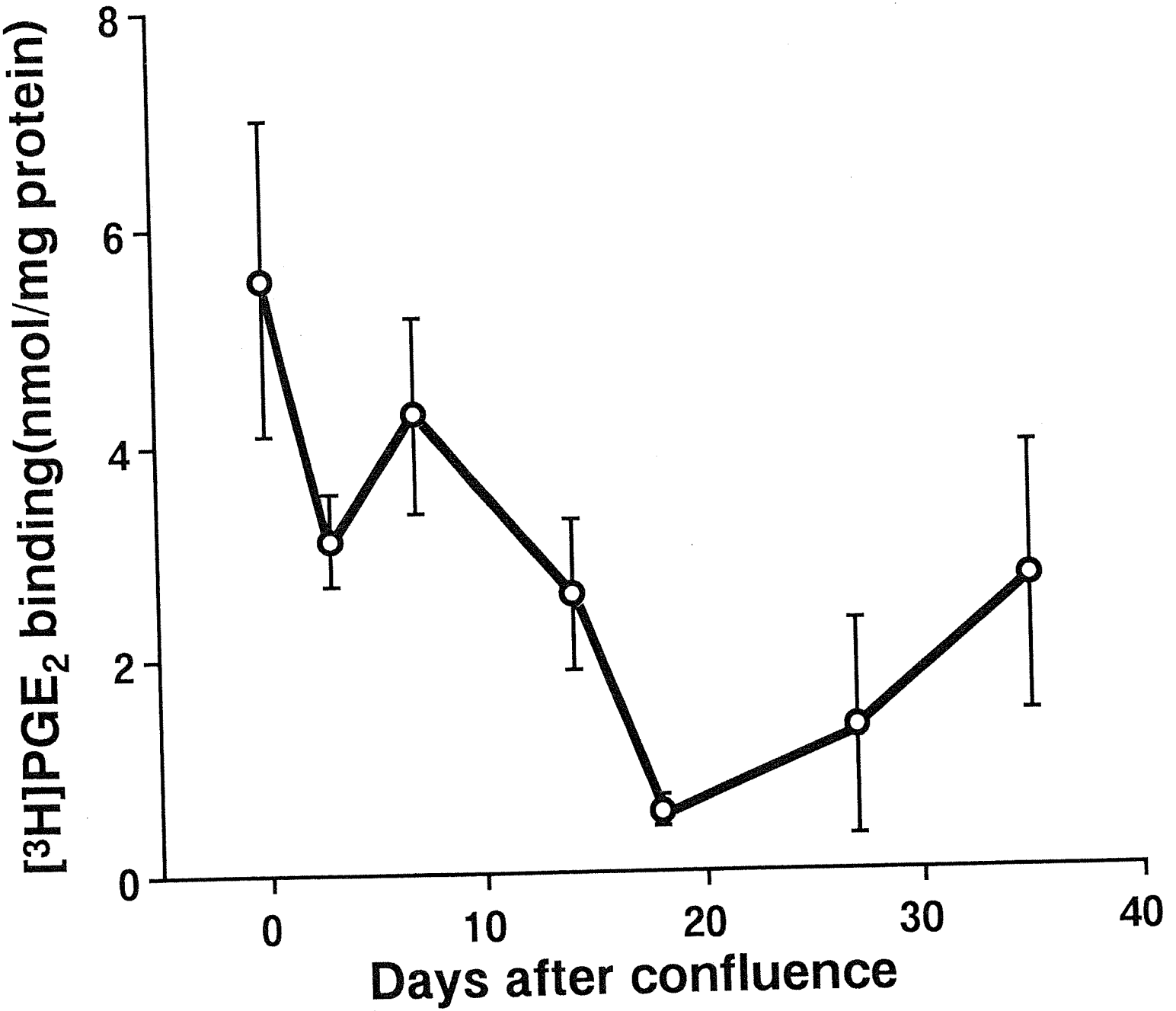
[3H]PGE2 binding(nmol/mg protein)
8
6
4
2
0
0
10
20
30
40
Days after confluence

Fig. 5