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Medium change amplifies mitogen-activated protein kinase-mediated prostaglandin E_2 synthesis in Swiss 3T3 fibroblasts

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Abstract

In Swiss 3T3 fibroblasts, changing the culture medium prior to stimulation resulted in an augmentation of bradykinin-induced prostaglandin E_2 synthesis. The augmentation depended on the duration of the exposure to the fresh medium, with a maximum effect at 1 h. Fetal calf serum in the fresh medium was essential for augmented prostaglandin E_2 synthesis. The medium change slightly augmented the bradykinin-induced increase in intracellular free Ca^{2+} concentration and phosphoinositide hydrolysis with a different time course from that for prostaglandin E_2 synthesis. 4′,5,7-Trihydroxyisoflavone (genistein) and 3,4-dihydroxybenzylidene-malononitrile (tyrphostin 23), inhibitors of tyrosine kinases, and 2′-amino-3′-methoxyflavone (PD98059), an inhibitor of mitogen-activated protein kinase (MAPK) kinase, attenuated the increase in prostaglandin E_2 synthesis. Bradykinin caused phosphorylation of cytosolic phospholipase A_2 and p42/p44 MAPK, which was augmented by the medium change. From the results, it is concluded that activation of MAPK and cytosolic phospholipase A_2 is involved in the augmentation of prostaglandin E_2 synthesis produced by the medium change.

Keywords: Medium change; Bradykinin; Prostaglandin E_2; Phospholipase A_2; Tyrosine kinase; Mitogen-activated protein kinase

1. Introduction

Prostanoid synthesis is regulated by two successive metabolic steps, the release of arachidonic acid from membrane phospholipids and its conversion to prostanoids (Flynn et al., 1981; Rosen et al., 1989). Phospholipase A_2, an enzyme responsible for arachidonic acid release, exists in two forms, secretory phospholipase A_2 and cytosolic phospholipase A_2 (Seilhsmer et al., 1989; Clark et al., 1991; Sharp et al., 1991; Kudo et al., 1993). Secretory phospholipase A_2 requires millimolar levels of Ca^{2+} for its activation and is divided into several groups, of which the pancreatic (type I) and nonpancreatic (type II) subtypes are well characterized (Kudo et al., 1993). Type II secretory phospholipase A_2 is widely distributed in several tissues and inflammatory exudates (Seilhsmer et al., 1989; Kudo et al., 1993). Cytosolic phospholipase A_2 is an intracellular enzyme that is found in various cells and tissues. Cytosolic phospholipase A_2 is characterized as a phospholipase A_2 involved in G-protein-coupled or tyrosine kinase-coupled receptor stimulation (Clark et al., 1991; Kim et al., 1991; Sharp et al., 1991). Recent evidence suggests that mitogen-activated protein kinase (MAPK) phosphorylates cytosolic phospholipase A_2, resulting in the release of arachidonic acid (Lin et al., 1993). Dual phosphorylation of tyrosine and serine/threonine residues is essential for MAPK activation.

Cyclooxygenase is the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids (Rosen et al., 1989). Two isoforms of cyclooxygenase exist, namely constitutive cyclooxygenase (cyclooxygenase-1) and inducible cyclooxygenase (cyclooxygenase-2) (Rosen et al., 1989; DeWitt, 1991; Kujubu et al., 1991; O'Banion et al.,

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1991). These glycosylated enzymes have 60% homology in nucleic acid and amino acid sequences and a similar molecular mass (70–74 kDa) (DeWitt, 1991). While cyclooxygenase-1 is stably expressed in cells, cyclooxygenase-2 is expressed after exposure to extracellular stimuli (Rosen et al., 1989; DeWitt, 1991; Kujubu et al., 1991; O'Banion et al., 1991).

Swiss 3T3 fibroblasts have the ability to synthesize prostaglandin E_2 in response to several stimuli, including bradykinin. We observed that changing the culture medium prior to stimulation with bradykinin accelerated prostaglandin E_2 synthesis. In the present study, we attempted to clarify the mechanism for the augmentation of prostaglandin E_2 synthesis in response to the change of culture medium. The results obtained suggest that MAPK has an important role in the augmentation.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and Eagle's minimum essential medium (EMEM) were purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal calf serum was obtained from JRH Biosciences (Lanexa, KS, USA). Bovine serum albumin and bradykinin were from Sigma Chemical (St. Louis, MO, USA). Prostaglandin E_2 and anti-prostaglandin E_2 antibody were generous gifts from Ono Pharmaceuticals (Osaka, Japan). 1-[2-(5′-Carboxyoxazol-2′-yl-6-aminobenzofuran-5-oxy)]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentapotassium salt (fura II-AM) was from Dojindo (Kumamoto, Japan). The anion exchange resin AG 1X-8 (formate form, 100–200 mesh) was from Bio-Rad (Richmond, CA, USA). [^{3}H]Prostaglandin E_2 (200 Ci/mmol), [^{3}H]inositol (16.5 Ci/mmol) and [^{32}P]phosphorus were from NEN/DuPont (Boston, MA, USA). Anti-phospho-MAPK antibody and 2′-Amino-3′-methoxyflavone (PD98059) were obtained from New England Biolabs (Beverly, MA, USA). Anti-cytosolic phospholipase A_2 antibody was from Santa Cruz (Delaware, CA, USA). Protein A Sepharose was from Zymed Laboratories (San Francisco, CA, USA). Triton X-100, 4′,5,7-trihydroxyisoflavone (genistein), 3,4-dihydroxybenzylidenemalononitrile (tyrphostin 23) and sodium dodecyl sulfate (SDS) were obtained from Wako Pure Chemicals (Tokyo, Japan). Collagenase was from Worthington Biochemical (Freehold, NJ, USA). Other chemicals and drugs were of reagent grade or of the highest quality available.

2.2. Cell culture

Swiss 3T3 fibroblasts were grown in DMEM containing 10% fetal calf serum, 50 units/ml of penicillin and 50 μg/ml of strepto-mycin in a 37°C humidified incubator under an atmosphere of 95% air and 5% CO_2.

2.3. Assay of prostaglandin E_2

Swiss 3T3 fibroblasts were seeded into 12-well plates at the density of 1.0×10^4 cells/well. The experiment was performed 2 days after cell seeding. The old culture medium was replaced by fresh culture medium, and the cells were incubated for 0.5–4 h. Then, the cells were washed twice with EMEM–HEPES (pH 7.35) and were incubated with bradykinin for 10 min after preincubation for 10 min. The medium was acidified to pH 4.0 by addition of 1 N HCl, and prostaglandin E_2 was extracted twice with ethyl acetate. After ethyl acetate was evaporated under a stream of N_2 gas, the sample was dissolved in 10 mM Tris–HCl (pH 7.6). Prostaglandin E_2 was determined by radioimmunoassay, as described previously (Nakahata et al., 1996). The sample was incubated overnight at 4°C with [^{3}H]prostaglandin E_2 (10,000 dpm) and anti-prostaglandin E_2 antibody (450 times dilution) in a final volume of 150 μl containing 0.5% bovine serum albumin in 100 mM Tris–HCl (pH 7.6). The free [^{3}H]prostaglandin E_2 was sedimented by centrifugation after addition of 0.5 ml of a buffer containing 0.5% charcoal, 0.075% dextran and 0.5% bovine serum albumin in 100 mM Tris–HCl (pH 7.6). The [^{3}H]prostaglandin E_2 bound to antibody was counted by liquid scintillation counting.

2.4. Measurement of intracellular free Ca^+ concentrations ($[Ca^{2+}]_i$) with fura II

Swiss 3T3 fibroblasts cultured in a 100-mm dish were incubated with fresh culture medium for 0.5–2 h. Then, the cells were washed with a modified Tyrode solution (final concentration, NaCl 137 mM, KCl 2.7 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 0.18 mM, NaH_2PO_4 0.4 mM, glucose 5.6 mM, HEPES 10 mM, pH 7.4) and were detached from the dish by treatment with 0.1% collagenase and 1.0% bovine serum albumin in 10 ml of the modified Tyrode solution. The cells were treated with 1 μM fura II-AM at 37°C for 15 min (Nakahata et al., 1994), centrifuged at 250 g for 1 min in order to remove the remaining fura II-AM, and then washed twice with the modified Tyrode solution. The cells were suspended in the modified Tyrode solution at a concentration of 10^6 cells/ml, and 2 ml of this suspension was used for the fura II assay. The fluorescence of fura II at 510 nm after excitation at 340 and 380 nm was recorded. The maximum fluorescence ratio was obtained in the presence of 0.1% Triton X-100, and the minimum fluorescence ratio was obtained in the presence of 2 mM EGTA. Free calcium concentrations were calculated by using the K_d of fura II for Ca^{2+} ions as 224 nM (Grynkiewicz et al., 1985).

2.5. Assay of [^{3}H]inositol phosphates

Phosphoinositide hydrolysis was monitored by determination of [^{3}H]inositol phosphates (Nakahata et al., 1990; Sakai et al., 1996). Swiss 3T3 fibroblasts were seeded into 6-well plates at the density of 3.0×10^4 cells/well. Two days after seeding, the medium was changed to DMEM containing 2 μCi/ml of [^{3}H]inositol for 18 h. Prior to assay, the cells were incubated with fresh culture medium containing 2 μCi/ml of [^{3}H]inositol for 0.5–2 h. The cells were washed twice with EMEM–HEPES (pH 7.35), and preincubated with EMEM–HEPES containing 10 mM LiCl. Ten minutes later, the reaction was started by the addition of bradykinin. The reaction was continued for an additional 10 min and was terminated by addition of 1 ml of 5% trichloroacetic acid after aspiration of the medium. The trichloroacetic acid extract was washed three times with ether and applied to an anion exchange column (AG 1X-8, formate form). Total [^{3}H]inositol phosphates were eluted with 1 M ammonium formate in 0.1 M formic acid and counted by liquid scintillation counting.

2.6. Immunoblotting of phospho-MAPK

Swiss 3T3 fibroblasts were seeded into 6-well plates at the density of 1.0×10^5 cells/well. Two days after seeding, the cells were incubated in fresh medium for 1 h. Then, the cells were washed twice with EMEM–HEPES (pH 7.35) and preincubated for 10 min at 37°C. After the cells were incubated with bradykinin for an additional 2 min, the reaction was terminated by the addition of Laemmli sample buffer (final concentration, Tris–HCl 187.5 mM, SDS 6%, glycerol 30%, 2-mercaptoethanol 15%, pH 6.8) after aspiration of the medium (Laemmli, 1970). The sample was boiled at 95°C for 5 min. Electrophoresis was performed on 11% acrylamide gels. Proteins were transferred electrically from the gel onto Immobilon polyvinylidene difluoride membranes (Millipore) by the semi-dry blotting method (Ohkubo et al., 1996). The immunoblots were blocked for 2 h with 2% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (TBST) at 25°C, and incubated with anti-phospho-MAPK antibody (rabbit) at 1 μg/ml for 2 h at 25°C. The immunoblots were washed several times and incubated with a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Ig) G in TBST containing 2% bovine serum albumin overnight at 4°C. Blots were developed by using a chemiluminescence assay kit (Bio Rad) and visualized by exposing the membrane to Hyperfilm ECL (Amersham).

2.7. Phosphorylation of cytosolic phospholipase A_2

Swiss 3T3 fibroblasts were seeded into 6-well plates at the density of 1.7×10^5 cells/well. Two days after seeding, the medium was changed to DMEM containing 200 μCi/ml of [^{32}P]phosphorus for 3 h. Prior to assay, the cells were incubated with fresh culture medium containing 200 μCi/ml of [^{32}P]phosphorus for 1 h. The cells were washed twice with EMEM–HEPES (pH 7.35) and preincubated for 10 min at 37°C. After the cells were incubated with bradykinin for an additional 2 min, the reaction was terminated by the addition of lysis buffer (final concentration, HEPES 15 mM, NaCl 140 mM, KCl 5.9 mM, $MgCl_2$ 1.2 mM, EDTA 10 mM, PMSF 2 mM, leupeptin 5 μg/ml, pepstatin A 1 μg/ml, sodium orthovanadate 0.2 mM, Nonidet P-40 1%) after aspiration of the medium (Gresham et al., 1996). The sample was sonicated and centrifuged at $15{,}000 \times g$ for 10 min to isolate solubilized enzyme. The supernatant was incubated with 10 μl (1 μg) of anti-cytosolic phospholipase A_2 antibody at 4°C for 1 h and then incubated at 4°C overnight after addition of 20 μl of protein A Sepharose. The sample was centrifuged at $15{,}000 \times g$ for 10 min to obtain immunoprecipitated materials. After extensive washing with lysis buffer, the final pellet was resuspended in Laemmli sample buffer. The sample was boiled at 95°C for 5 min. Electrophoresis was performed on 8% acrylamide gels. The relative intensity of the bands observed in autoradiographs of the immunoprecipitated material was analyzed by using a molecular imager (GS363, Bio-Rad, Hercules, CA).

2.8. Data analysis

The results obtained are expressed as means with S.E.M., and the significance of differences ($P < 0.05$) was analyzed by analysis of variance (ANOVA).

3. Results

3.1. Effect of medium change on prostaglandin E_2 synthesis stimulated by bradykinin in Swiss 3T3 fibroblasts

While bradykinin caused prostaglandin E_2 synthesis to a small extent in Swiss 3T3 fibroblasts, the medium change prior to the stimulation augmented bradykinin-induced prostaglandin E_2 synthesis (Fig. 1). When the medium was changed 1 h before the stimulation, prostaglandin E_2 synthesis appeared to be maximum. The cells were cultured with different concentrations of fetal calf serum for 2 days, and then the medium was changed to DMEM containing the same concentration of fetal calf serum (Fig. 2). Since the cells grew differently in the culture medium containing different concentrations of fetal calf serum, the results for prostaglandin E_2 synthesis were normalized to the cell counts. Basal prostaglandin E_2 synthesis when the medium was not changed was dependent on the fetal calf serum concentration in the culture medium. Bradykinin only slightly stimulated prostaglandin E_2 synthesis when the medium was not changed. The bradykinin-induced augmentation of prostaglandin E_2 synthesis after the medium change was clearly dependent on the fetal calf serum concentration in the culture medium. In other words, fetal

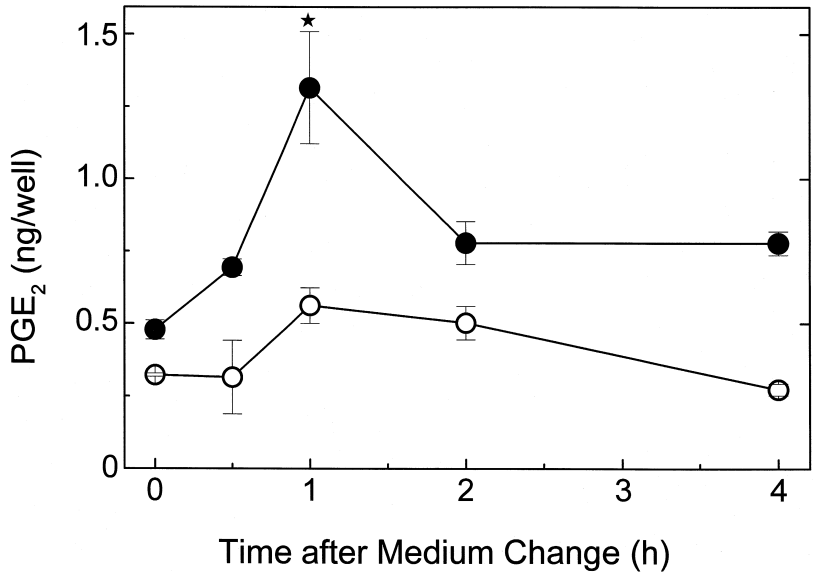

Fig. 1. Effect of medium change on the bradykinin-induced prostaglandin E_2 synthesis. The culture medium was changed to fresh culture medium, and the cells were incubated for 0.5–4 h. The cells were incubated with (●) or without (○) 1 μM bradykinin for 10 min. Determination of prostaglandin E_2 was as described in Section 2. Each point represents the mean with S.E.M. from three determinations. ★Significant difference from no medium change ($P < 0.05$). The data are representative of three experiments with similar results.

calf serum contains a factor(s) that affects prostaglandin E_2 synthesis.

3.2. Effect of medium change on bradykinin-induced increase in $[Ca^{2+}]_i$

Arachidonic acid, a precursor of prostaglandin E_2, is liberated from phospholipids by activation of phospholipase A_2 (Chen et al., 1997). An increase in $[Ca^{2+}]_i$ is thought to be important for the activation of phospholipase A_2 (Murthy et al., 1995). Therefore, we determined $[Ca^{2+}]_i$ after the medium change. Bradykinin caused a transient increase in $[Ca^{2+}]_i$ with a peak 20–30 s after its addition in fura II-loaded cells (data not shown). Bradykinin increased $[Ca^{2+}]_i$ in cells in a concentration-dependent manner when the medium was not changed (Fig. 3). The

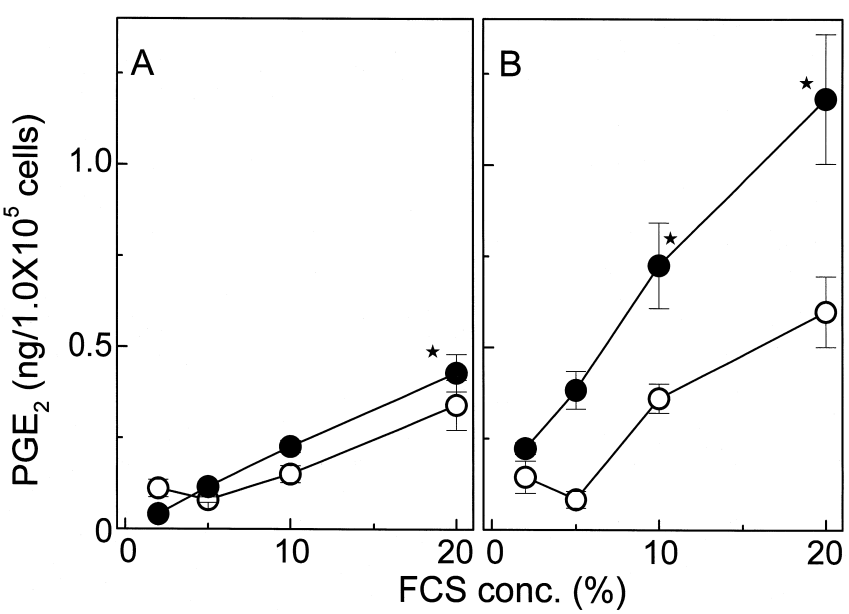

Fig. 2. Fetal calf serum dependence of bradykinin-induced prostaglandin E_2 synthesis. (A) The cells were cultured with different concentrations of fetal calf serum for 2 days. The experiments were performed without a change of medium. (B) The cells were cultured with different concentrations of fetal calf serum for 2 days and were then cultured for 1 h after changing the medium to DMEM containing the same fetal calf serum concentration. After the cells were washed two times with EMEM–HEPES, they were incubated with (●) or without (○) 1 μM bradykinin for 10 min. The results were normalized to the cell counts. Each point represents the mean with S.E.M. from three determinations. ★Significant difference from no medium change ($P < 0.05$).

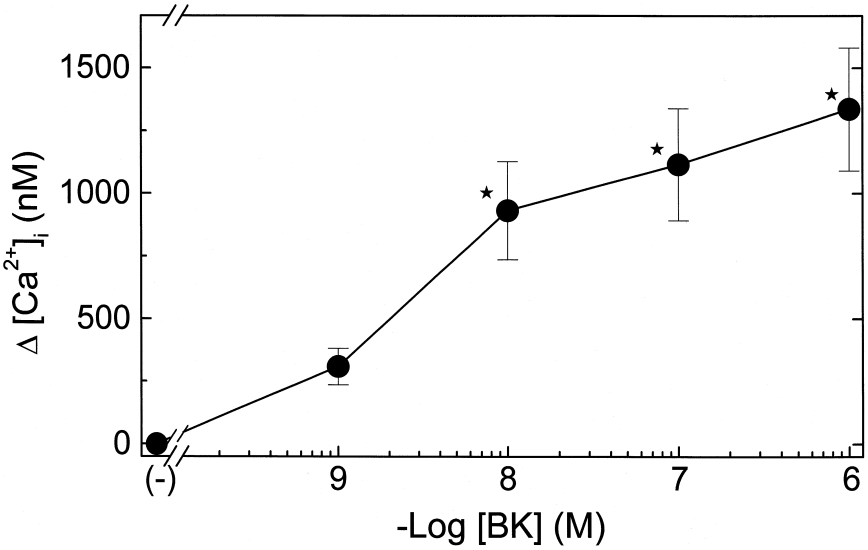

Fig. 3. Effect of bradykinin on the increase in $[Ca^{2+}]_i$. Cells loaded with fura II were suspended in the modified Tyrode solution. Determination of $[Ca^{2+}]_i$ was as described in Section 2. Bradykinin-induced increase in $[Ca^{2+}]_i$ from basal level is expressed as $\Delta[Ca^{2+}]_i$. Each point represents the mean with S.E.M. from four determinations. ★Significant difference from no medium change ($P < 0.05$).

medium change facilitated a slight increase in $[Ca^{2+}]_i$ induced by 1 μM bradykinin (Fig. 4). The medium change-induced facilitation of the Ca^{2+} increase was weaker than the augmentation of prostaglandin E_2 synthesis. In addition, the time course of the increase in $[Ca^{2+}]_i$ was different from that of the augmentation of prostaglandin E_2 synthesis. These results indicate that the increase in $[Ca^{2+}]_i$ does not make a major contribution to the augmentation of prostaglandin E_2 synthesis seen after the medium was changed.

3.3. Effect of medium change on bradykinin-induced phosphoinositide hydrolysis

It is known that bradykinin activates phospholipase C, which produces inositol 1,4,5-trisphosphate (IP_3) and dia-

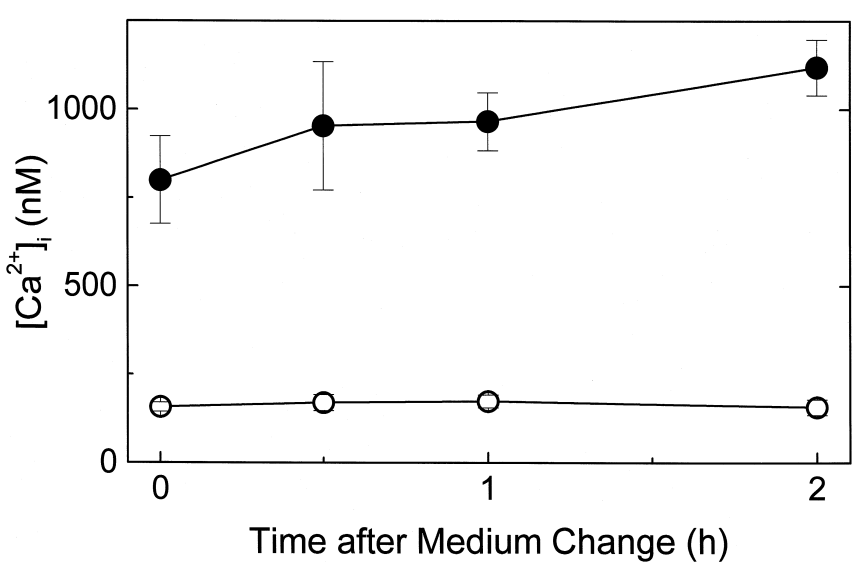

Fig. 4. Effect of medium change on the bradykinin-induced increase in $[Ca^{2+}]_i$. The culture medium was changed to fresh culture medium, and the cells were incubated for 0.5–2 h. Then, the cells were loaded with fura II and suspended in modified Tyrode solution. Results are shown as resting $[Ca^{2+}]_i$ level (○) and 1 μM bradykinin-induced $[Ca^{2+}]_i$ level (●). Each point represents the mean with S.E.M. from four determinations.

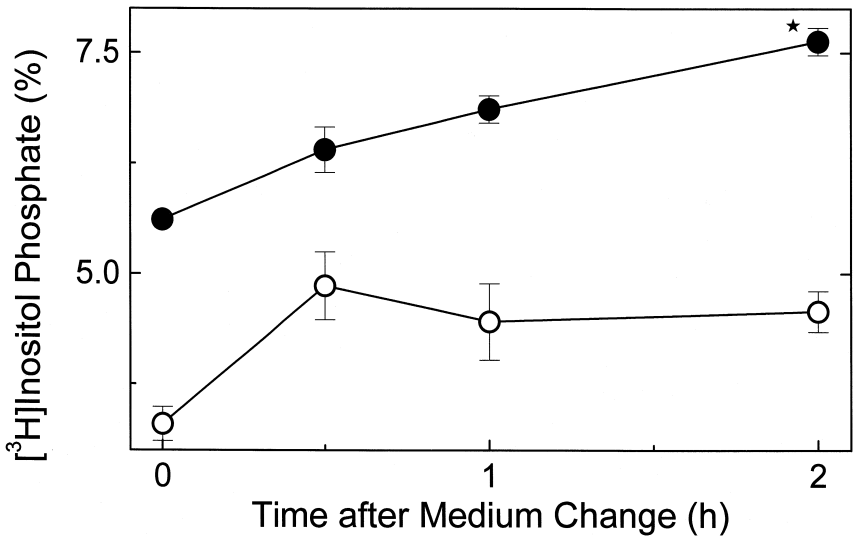

Fig. 5. Effect of medium change on phosphoinositide hydrolysis elicited by bradykinin. The cells were labeled with 2 μCi/ml of [^{3}H]inositol for 18 h. Prior to assay, the cells were incubated with fresh culture medium containing 2 μCi/ml of [^{3}H]inositol for 0.5–2 h. After being washed, the cells were incubated in the presence (●) or absence (○) of 1 μM bradykinin for 10 min. Analysis of [^{3}H]inositol phosphates (IP) was as described in Section 2. Accumulated [^{3}H]inositol phosphates are expressed as percentage of [^{3}H]phosphoinositide (PI) pool. [^{3}H]PI pools are: 0 h, 33,495.08 dpm/well; 0.5 h, 26,272.55 dpm/well; 1 h, 30,064.23 dpm/well; 2 h, 26,693.08 dpm/well without stimulation of bradykinin, and 0 h, 31,339.12 dpm/well; 0.5 h, 25,290.84 dpm/well; 1 h, 25,641.23 dpm/well; 2 h, 29,087.87 dpm/well with stimulation of bradykinin. Each point represents the mean with S.E.M. from three determinations. ★Significant difference from no medium change ($P < 0.05$). The data are representative of three experiments with similar results.

cylglycerol (Nishizawa, 1984; Ogawa et al., 1995). Generated IP_3 releases Ca^{2+} from its storage site, resulting in an increase in $[Ca^{2+}]_i$. We examined phosphoinositide hydrolysis in cells labeled with [^{3}H]inositol (Fig. 5). To normalize the labeling efficiency and cell counts, the results are expressed as the percentage of accumulated [^{3}H]inositol

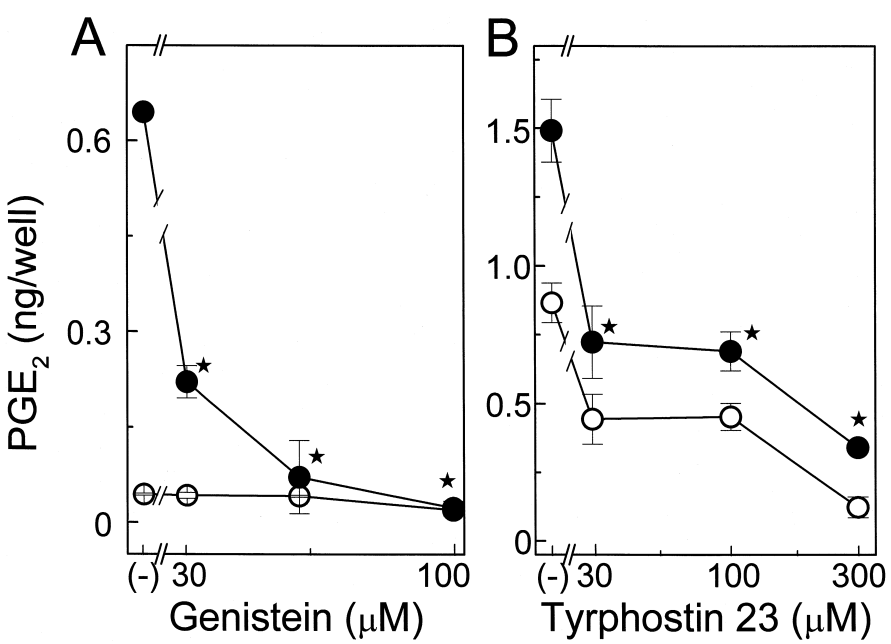

Fig. 6. Effects of genistein (A) and tyrphostin 23 (B) on the augmentation of bradykinin-induced prostaglandin E_2 synthesis produced by changing the medium. The cells were treated with genistein (10–100 μM) or tyrphostin 23 (30–300 μM) during the incubation with fresh medium for 1 h and incubation with (●) or without (○) 1 μM bradykinin for 10 min. Note that genistein and tyrphostin 23 attenuated bradykinin-induced prostaglandin E_2 synthesis. Each point represents the mean with S.E.M. from three determinations. ★Significant difference from no medium change ($P < 0.05$). The data are representative of three experiments with similar results.

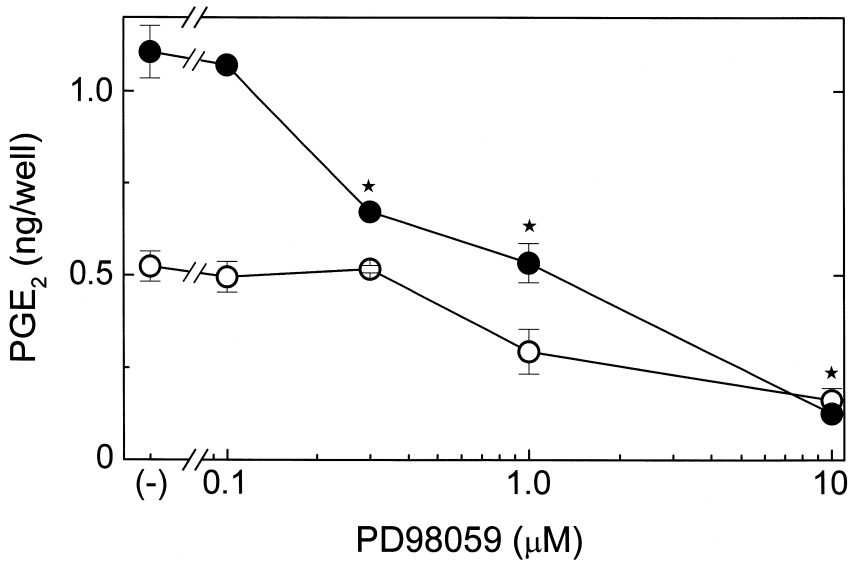

Fig. 7. Effect of PD98059 on the augmentation of bradykinin-induced prostaglandin E_2 synthesis produced by changing the medium. The cells were treated with PD98059 at concentrations of 0.1–10 μM for medium change and assay. Vehicle: (○); 1 μM bradykinin: (●). Note that PD98059 attenuated bradykinin-induced prostaglandin E_2 synthesis. Each point represents the mean with S.E.M. from three determinations. ★Significant difference from no medium change ($P < 0.05$). The data are representative of three experiments with similar results.

phosphates to total [^{3}H]phosphoinositides. The basal- and bradykinin-induced accumulation of [^{3}H]inositol phosphate was slightly increased by the medium change. The increase was dependent on when the medium was changed. The time course of the increase corresponded to that for the increase in $[Ca^{2+}]_i$ after the medium change, but was quite different from that for the augmentation of prostaglandin E_2 synthesis. The activation of phospholipase C or $[Ca^{2+}]_i$ by the medium change may not be a main cause of the augmentation of prostaglandin E_2 synthesis.

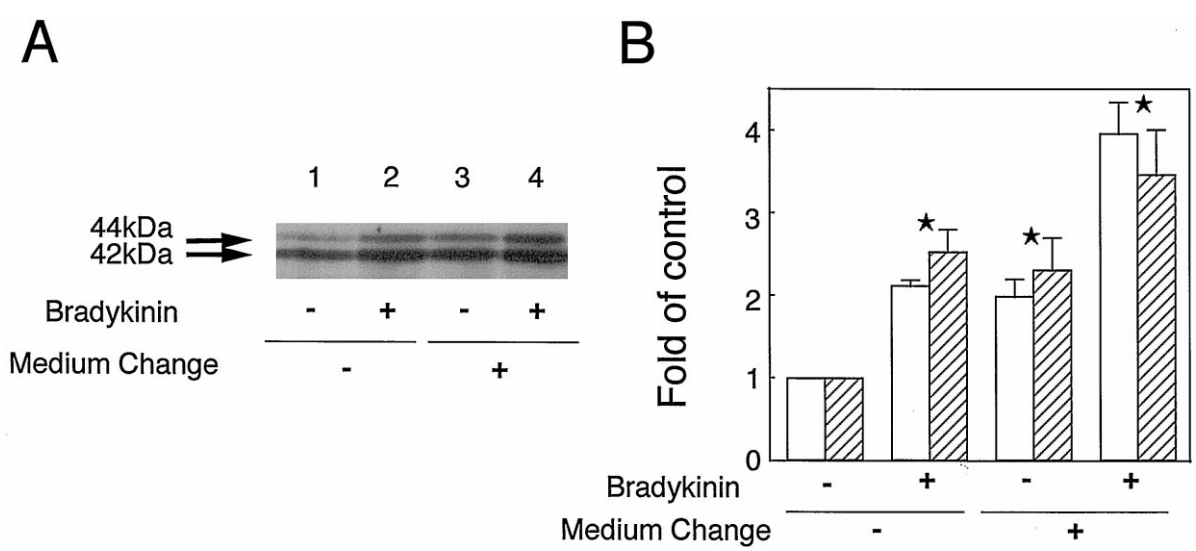

Fig. 8. Effect of medium change on MAPK phosphorylation. Prior to assay, the cells were incubated with fresh culture medium for 1 h. After being washed, the cells were incubated in the presence or absence of 1 μM bradykinin for 2 min. The proteins were resolved by 11% SDS-polyacrylamide gel electrophoresis and immunoblotted by using anti-phospho-MAPK antibody for 2 h at 25°C. Analytical method of phosphorylation of phospho-MAPK was as described in Section 2. (A) Immunoblotting (B) Densitometric analysis of the blots. Open column: the phosphorylation of p44 MAPK; hatched column: the phosphorylation of p42 MAPK. The results are shown as the fold increase from control (without medium change or bradykinin). Each column represents the mean value with S.E.M. from three experiments. ★Significant difference from no medium change ($P < 0.05$).

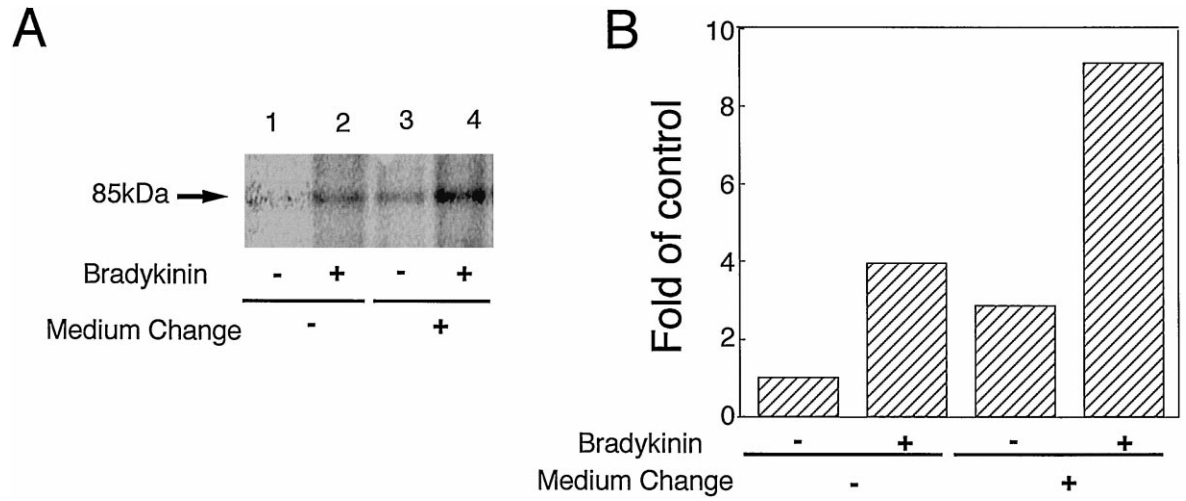

Fig. 9. Effect of medium change on cytosolic phospholipase A_2 phosphorylation. Prior to assay, the cells were incubated with fresh culture medium for 1 h. After being washed, the cells were incubated in the presence or absence of 1 μM bradykinin for 2 min. The sample was immunoprecipitated with an anti-cytosolic phospholipase A_2 antibody. The immunoprecipitate was fractionated on a 8% SDS-polyacrylamide gel by electrophoresis. (A) The relative intensity of the bands observed in autoradiographs of the immunoprecipitated material were analyzed by using a molecular imager (GS363, Bio-Rad). (B) Densitometric analysis of the blots. The results are shown as the fold increase from control (without medium change or bradykinin). The data are representative of two experiments with similar results.

3.4. Effects of genistein and tyrphostin 23, inhibitors of protein tyrosine kinases, and PD98059, an inhibitor of MAPK kinase, on the augmentation of prostaglandin E_2 synthesis by medium change

Recent evidence suggests that tyrosine kinase is important for the activation of cytosolic phospholipase A_2 (Zor et al., 1993). Therefore, we examined the effect of genistein and tyrphostin 23, inhibitors of protein tyrosine kinases, on the medium change-induced augmentation of prostaglandin E_2 synthesis. The treatment of the cells with genistein or tyrphostin 23 for the entire period after the medium change resulted in the inhibition of prostaglandin E_2 synthesis in a concentration-dependent manner (Fig. 6), suggesting that protein tyrosine kinases might have an important role in prosta-glandin E_2 synthesis. The MAPK family of serine/threonine protein kinases is known to be involved in a wide range of cellular functions, including cytosolic phospholipase A_2 activation (Pumiglia and Decker, 1997). Therefore, we examined the effect of PD98059, a specific inhibitor of the MAPK kinase (MEK) (Pang et al., 1995), on the augmentation of bradykinin-induced prostaglandin E_2 synthesis. PD98059 clearly inhibited prostaglandin E_2 synthesis in a concentration-dependent manner (Fig. 7), demonstrating that MAPK plays a role in the augmentation of prostaglandin E_2 synthesis.

3.5. Effect of medium change on MAPK and cytosolic phospholipase A_2 phosphorylation

Genistein and PD98059 inhibited the augmentation of prostaglandin E_2 synthesis produced by the medium change in a concentration-dependent manner. Therefore, we examined protein phosphorylation by immunoblotting, using an anti-phospho-MAPK antibody (Fig. 8). When the cells were incubated in fresh medium for 1 h, the phosphorylation of p42/p44 MAPK was markedly activated by bradykinin. MAPK is known to cause the phosphorylation of cytosolic phospholipase A_2, resulting in the release of arachidonic acid (Lin et al., 1993). Thus, we examined protein phosphorylation by immunoprecipitation, using an anti-cytosolic phospholipase A_2 antibody and [^{32}P]phosphorus (Fig. 9). The bradykinin-induced phosphorylation of cytosolic phospholipase A_2 was markedly activated by the medium change, suggesting that cytosolic phospholipase A_2 is involved in the augmentation of prostaglandin E_2 synthesis seen after the medium was changed.

4. Discussion

When bradykinin binds to the B_2 type of receptor, it stimulates the release of arachidonic acid in Swiss 3T3 fibroblasts by activating phospholipase A_2 through a G protein (Schrör, 1992; Kramer et al., 1996), which is pertussis toxin-sensitive, probably Gi (Kikuchi et al., 1986). Arachidonic acid is mainly converted to prostaglandin E_2 in these cells. Under normal culture conditions (DMEM-10% fetal calf serum), however, Swiss 3T3 cells produce only a small amount of prostaglandin E_2 in response to bradykinin. Searching for optimal culture conditions, we found that a change of culture medium prior to bradykinin-stimulation promoted prostaglandin E_2 synthesis. The augmented prostaglandin E_2 synthesis was dependent on the duration of exposure of the cells to the fresh

culture medium, with an optimal period of 1 h. These findings are important for clarifying the regulatory mechanisms of the arachidonic acid cascade by external stimuli.

Bradykinin-induced prostaglandin E_2 synthesis was dependent on the concentration of fetal calf serum in the culture medium. Therefore, it is assumed that fetal calf serum is essential for augmented prostaglandin E_2 synthesis. Since fetal calf serum contains many growth factors (Edwin and Mitchell, 1992), it is assumed that some growth factor(s) in fetal calf serum might have a key role in the augmentation of prostaglandin E_2 synthesis. It has been shown that an increase in $[Ca^{2+}]_i$ is critical for activation of cytosolic phospholipase A_2 (Nishizawa, 1984). The medium change slightly augmented the bradykinin-induced increase in $[Ca^{2+}]_i$ in fura II-loaded Swiss 3T3 cells. However, time-course analysis revealed that the timing of the change in $[Ca^{2+}]_i$ did not correspond to the timing of the augmentation of prostaglandin E_2 synthesis. In agreement with the Ca^{2+} response, the timing of the increase in phosphoinositide hydrolysis was not comparable to that for the augmentation of prostaglandin E_2 synthesis produced by the medium change.

Tyrosine-specific protein kinase activity is associated with the cellular receptors for several growth factors such as EGF, platelet-derived growth factor (PDGF), insulin, and insulin-like growth factor I (IGF I) (Jerome et al., 1991). Tyrosine phosphorylation mediates cell proliferation and cell transformation (Li et al., 1993). It is reported that tyrosine kinase is also important for the activation of phospholipase A_2 (Goldman et al., 1994). In fact, genistein and tyrphostin 23, inhibitors of tyrosine-specific protein kinases, inhibited the augmentation of bradykinin-induced prostaglandin E_2 synthesis produced by the medium change in a concentration-dependent manner. Therefore, it is assumed that tyrosine phosphorylation is involved in the augmentation.

It has been shown that purified cytosolic phospholipase A_2 can be stoichiometrically phosphorylated by MAPK (Lin et al., 1993). This phosphorylation event stimulates cytosolic phospholipase A_2 activity (Sa et al., 1995). PD98059, a MAPK kinase (MEK) inhibitor, clearly inhibited the augmented prostaglandin E_2 synthesis induced by bradykinin after the medium change. Furthermore, the bradykinin-induced phosphorylation of p42/p44 MAP kinases was facilitated by the medium change. In addition, the bradykinin-induced phosphorylation of cytosolic phospholipase A_2 was also augmented by the medium change. These results suggest that the augmentation of prostaglandin E_2 synthesis produced by changing the culture medium is mediated through the phosphorylation and activation of MAPK and cytosolic phospholipase A_2.

In this study, we demonstrated that changing the medium prior to stimulation resulted in the augmentation of the bradykinin-induced activation of arachidonic acid metabolism through MAPK-phospholipase A_2. Since fetal calf serum was essential for the augmentation, it apparently contains a factor(s) that activates the arachidonic acid cascade. These results are important for clarifying the regulation of the arachidonic acid cascade. The further analysis of the augmentation produced by the medium change or the factor(s) involved will be helpful in understanding the molecular regulatory mechanism of the arachidonic acid cascade.

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