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ENHANCEMENT OF BRADYKININ-INDUCED PROSTACYCLIN SYNTHESIS IN PORCINE AORTIC ENDOTHELIAL CELLS BY PERTUSSIS TOXIN

POSSIBLE IMPLICATION OF LIPOCORTIN I

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Abstract—Bradykinin-stimulated prostacyclin synthesis in porcine aortic endothelial cells was enhanced by pretreatment of the cells with pertussis toxin or islet-activating protein (IAP) for 5 hr or longer. Although ADP-ribosylation of a protein with a molecular weight of 41–42 kD in the cell membranes was completed by 3 hr after the addition of IAP into the incubation medium, there was good correlation between enhancement of bradykinin-induced prostacyclin synthesis and ADP-ribosylation of the IAP substrate over a wide range of IAP concentrations. Furthermore, even if IAP was removed from the incubation medium at 3 hr, bradykinin-induced prostaglandin synthesis at 24 hr was still potentiated. Cycloheximide and actinomycin D enhanced bradykinin-induced prostacyclin synthesis and apparently blocked the effect of IAP. Since this result suggested the involvement of an inhibitor protein(s) of prostacyclin synthesis in the IAP effect, we studied the effect of IAP on the level of lipocortin I which is known to inhibit phospholipase A_2. Western and Northern blot analyses revealed that IAP decreased the amounts of protein and mRNA of lipocortin I. These results suggest that the enhancement of bradykinin-induced prostacyclin synthesis by IAP is associated with a decrease in the level of lipocortin I.

The generation of prostacyclin, which inhibits platelet aggregation and relaxes vascular smooth muscle, is one of the most important functions of vascular endothelial cells. Release of free arachidonate, a rate-limiting process of prostacyclin synthesis, is thought to be catalyzed mainly by phospholipase A_2 [1–4]. It has often been postulated that activation of phospholipase A_2 is the consequence of phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate in endothelial cells from blood vessels because Ca^{2+} mobilized by inositol trisphosphate (IP_3) can stimulate phospholipase A_2 [4–6]. However, recent studies have demonstrated that bradykinin-induced activation of phospholipase A_2 is independent of the activation of polyphosphoinositide-hydrolyzing phospholipase C [2, 7].

It is suggested that GTP binding proteins (G-proteins) transduce receptor-mediated signals for phospholipase A_2 activation in a variety of cells [8–12]. Pertussis toxin or islet-activating protein (IAP) inactivates some G-proteins such as G_i and G_o by ADP-ribosylating their α-subunit. The effect of IAP on prostacyclin synthesis has been controversial [13–16]. Clark *et al.* [13] demonstrated that IAP inhibits prostacyclin synthesis stimulated by leukotrienes and tumor necrosis factor but not that stimulated by bradykinin or calcium ionophore in bovine aortic endothelial cells. They suggested that an IAP substrate (possibly G_i) is involved in the induction of phospholipase A_2-activating protein [14]. Angiogenin-stimulated prostacyclin synthesis in bovine adrenal capillary endothelial cells is also inhibited by IAP [15]. On the other hand, Pirotton *et al.* [16] demonstrated that IAP enhances ATP-induced prostacyclin release from bovine aortic endothelial cells, but they did not comment on the mechanism of IAP action. In a murine macrophage cell line, Burch *et al.* [17] found that short incubations (2–4 hr) of the cells with IAP stimulated prostaglandin E_2 synthesis accompanying ADP-ribosylation of a protein with molecular weight (M_r) = 41 kD, and thereby proposed that phospholipase A_2, like adenylate cyclase, is regulated by both stimulatory and inhibitory G-proteins.

The purpose of the present study was to shed new light on the effect of IAP on prostacyclin synthesis. Here we show that the enhancing effect of IAP on bradykinin-induced prostacyclin synthesis in porcine aortic endothelial cells (PAEC) was closely associated with the preceding ADP-ribosylation of a membrane protein with M_r = 41–42 kD and with the decrease in the amounts of protein and mRNA of lipocortin I.

MATERIALS AND METHODS

Cell culture. PAEC were isolated from fresh pig aortae obtained from a local slaughterhouse by scraping the luminal surface with a razor blade as described by Wey *et al.* [18] and maintained in medium 199 (Gibco) supplemented with 20% fetal calf serum (Gibco), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer

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(pH 7.4), and antibiotics. The cells were characterized by (i) a polygonal shape, (ii) the presence of Weibel–Palade bodies as observed by electron microscopy, and (iii) immunohistochemical studies of factor VIII-related antigen using an avidin/biotin immunoperoxidase test system (Lipshaw/Immunon). Experiments were performed with cells between passages 3 and 6. In some experiments, the confluent cells were cultured with pertussis toxin, cycloheximide or actinomycin D for the indicated periods. Cell viability (trypan blue exclusion) was always 100% after these treatments.

Prostacyclin synthesis. Confluent PAEC in 12-well plates were used for the experiments. Before an experiment, media were gently aspirated and the wells were washed with 0.5 mL of Medium 199 including 10 mM Hepes buffer (pH 7.4) (199-Hepes). This medium was gently aspirated and replaced with 0.5 mL of 199-Hepes. The cells were incubated for 15 min at 37°; then bradykinin or other stimulants were added, and the cells were incubated for an additional 5 min at 37°. The media were directly assayed for 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), a stable metabolite of prostacyclin, using a radioimmunoassay kit (New England Nuclear).

Formation of [^{3}H]*inositol phosphates.* PAEC in 6-well plates were labeled for 96 hr with *myo*-[^{3}H]inositol (10 μCi/well) in growth medium. The confluent cells were incubated for 15 min at 37° in 199-Hepes containing 10 mM LiCl after one washing; then bradykinin was added, and the cells were incubated for 1 min at 37°. The media were aspirated before the reactions were stopped by addition of 1 mL of ice-cold methanol. After scraping of the cells and transfer of the samples to a centrifuge tube, chloroform and water were added to a final ratio of chloroform : methanol : H_2O of 1 : 1 : 0.8. The aqueous upper phase was applied to Dowex columns to separate inositol phosphates according to the methods of Slivka and Insel [19]. Inositol mono-, bis-, and trisphosphate (IP_1, IP_2, and IP_3 respectively) were eluted with 0.15 M $HCOONH_4$ in 0.1 M HCOOH (10 mL), 0.4 M $HCOONH_4$ in 0.1 M HCOOH (10 mL), and 2 M $HCOONH_4$ in 0.1 M HCOOH (5 mL) respectively.

[^{32}P]*ADP-ribosylation.* Endogenous ADP-ribosylation of IAP substrates was estimated by the decrease in *in vitro* [^{32}P]ADP-ribosylation of the membranes prepared from IAP-treated PAEC. Confluent PAEC in 175 cm^2 flasks were washed in phosphate-buffered saline (PBS) and scraped with a rubber policeman. The cell pellets were lysed by freezing and thawing and then resuspended in PBS containing 1 mM EDTA. Cell lysates were homogenized and centrifuged at 30,000 *g* for 20 min. The resulting membrane pellets were resuspended and incubated with 6.5 μM [^{32}P]NAD and 20 μg/mL of the preactivated IAP at 30° for 20 min in 0.1 M potassium phosphate (pH 7.5) containing 20 mM thymidine, 2.5 mM $MgCl_2$, and 1 mM ATP in a final volume of 110 μL. The preactivation of IAP was done by incubation with 10 mM dithiothreitol in potassium phosphate buffer (pH 7.5) at 30° for 30 min [10]. This converted disulfide bond in the A-protomer of IAP to sulfhydryl groups. The radiolabeled membranes were washed, solubilized in the Laemmli sample buffer [20], and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [20]. Autoradiography was performed using Fuji HR-A. The autoradiograph signals of ADP-ribosylation of a protein with M_r = 41–42 kD were quantitated by a scanning densitometer (Biomed Instruments, Inc., Fullerton, CA, U.S.A.) and integration of peak areas.

Isolation of mRNA. Crude total RNAs were extracted from PAEC by the acid–guanidium thiocyanate–phenol–chloroform (AGPC) method [21].

Northern blot analysis. Ten micrograms of total RNA from PAEC was electrophoresed on a 1% formaldehyde agarose gel and transferred to a nitrocellulose filter. Prehybridization and hybridization were done as described in Ref. 22 using an EcoRI-BglII 0.42 kilobase pairs (kbp) fragment of mouse lipocortin cDNA [23] as a probe which was labeled with [α-^{32}P]dCTP by the random prime labeling method (sp. act. *ca.* 10^9 cpm/μg). Then the lipocortin I probe was removed from the filter and re-hybridized with mouse β-actin cDNA pAL41 [24], a 1.1 kbp fragment provided by Dr. S. Toyama (Kyoto University, Kyoto, Japan), as a probe to ensure equivalent amounts of RNA on the gel.

Western blot analysis. Lipocortin I protein molecules were analyzed on SDS–PAGE (15% gel) according to the method of Laemmli [20], and the gel was stained with Coomassie brilliant blue. Otherwise, Western blot analysis was carried out according to Burnett [25] after SDS–PAGE. Rabbit anti-rat lipocortin I antiserum [26] was used as a first antibody, and alkaline phosphatase conjugated goat anti-rabbit IgG as a second antibody. The staining was performed with a color developer (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) under the conditions recommended in the instruction manual.

Statistical analysis. In most cases, values are expressed as means ± SD. Student's *t*-test (two-tailed) was used to determine whether a significant difference existed between two mean values.

Materials. IAP was obtained from List Biological Laboratories. Bradykinin and vasopressin were obtained from Peptide Institute (Osaka, Japan). A23187 and arachidonic acid were purchased from Sigma. [^{32}P]NAD (1.08 TBq/mmol) and *myo*-[^{3}H]inositol (1.51 TBq/mmol) were from New England Nuclear. Restriction enzymes were purchased from the Takara-Shuzo Co., Ltd. (Kyoto, Japan) and were used according to the manufacturer's instructions. A random priming kit and [α-^{32}P]dCTP (sp. act. *ca.* 185 TBq/mmol) were purchased from Amersham. Alkaline phosphatase conjugated goat anti-rabbit IgG and a color developer were obtained from Promega (Madison, WI, U.S.A.).

RESULTS

Effects of neomycin on bradykinin-stimulated syntheses of prostacyclin and inositol phosphates. Bradykinin time- and concentration-dependently stimulates the synthesis of prostacyclin and inositol phosphates in PAEC, as described by other investigators [5, 7]. A significant increase in IP_3 production reached a maximum value between 30 and 60 sec, whereas 6-keto $PGF_{1\alpha}$, a stable metabolite

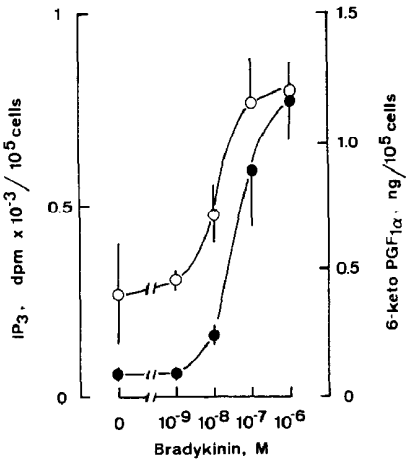

Fig. 1. Concentration–response curve for bradykinin-stimulated prostacyclin and IP_3 syntheses in PAEC. Prostacyclin (●) and IP_3 (○) were determined as described in Materials and Methods. Values are the means ± SD for three determinations.

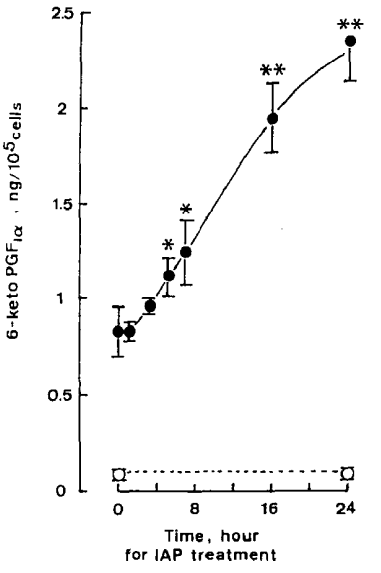

Fig. 2. Stimulation of bradykinin-induced prostacyclin synthesis by IAP treatment of PAEC. After the cells reached confluence, IAP (100 ng/mL) was added to the medium at 24, 16, 7, 5, 3, and 1 hr before assay for prostacyclin synthesis. Cells were washed and incubated with (●) or without (○) 1 μM bradykinin for 5 min. The media were assayed for 6-keto $PGF_{1\alpha}$. Values are the means ± SD for three determinations. Key: (*) $P < 0.05$ and (**) $P < 0.005$ compared to IAP-free.

of prostacyclin, was released and accumulated in the medium, reaching a maximum value at 5 min (results not shown). The effects of bradykinin on prostacyclin and IP_3 syntheses were concentration dependent over the range of 10^{-9}–10^{-6} M with EC_{50} values of 3×10^{-8} and 1×10^{-8} M respectively (Fig. 1). The aminoglycoside antibiotic neomycin, which is reported to bind polyphosphoinositides [27] and to inhibit phosphatidyl inositol-specific phospholipase C [19, 28], inhibited bradykinin-induced accumulation of IP_3, but not of IP_1, IP_2 or prostacyclin (Table 1).

Time course of IAP-induced changes of prostacyclin synthesis and ADP-ribosylation of a membrane protein. Although the treatment of PAEC with IAP for up to 3 hr had no effect on prostacyclin synthesis, prolonging the treatment period gave rise to time-dependent potentiation of bradykinin-induced prostacyclin synthesis (Fig. 2). However, IAP had no effect on basal prostacyclin synthesis. Next, we examined ADP-ribosylation of proteins in PAEC membranes, since the molecular basis of IAP action is thought to be a toxin-catalyzed ADP-ribosylation of G-proteins [10, 29, 30]. Incubation of the PAEC membranes with [^{32}P]NAD and preactivated IAP resulted in incorporation of the radioactivity into two proteins with M_r values of 29–30 and 41–42 kD, of which only the labeling of the substrate with M_r = 41–42 kD was inhibited by preincubation of the cells with IAP (Fig. 3). This inhibition is because the sites utilized by the labeled NAD had already been ribosylated via endogenous NAD. The inhibition had been accomplished at 3 hr, when IAP had no effect on bradykinin-burst prostacyclin synthesis.

ADP-ribosylation of a membrane protein by IAP as correlated with its stimulatory action on bradykinin-induced prostacyclin synthesis. As shown in Fig. 4, the stimulatory effect of IAP was observed at

Table 1. Effect of neomycin on prostacyclin and inositol phosphate productions in PAEC

Treatment	BK	6-keto $PGF_{1\alpha}$ (ng/10^5 cells)	Inositol phosphates (dpm/10^5 cells)		
			IP_1	IP_2	IP_3
Control	−	0.16 ± 0.05	858 ± 19	167 ± 111	175 ± 34
	+	1.04 ± 0.15	1413 ± 105	438 ± 80	743 ± 158
Neomycin	−	0.09 ± 0.03	781 ± 56	217 ± 86	243 ± 182
	+	1.16 ± 0.03	1363 ± 62	509 ± 74	300 ± 49*

PAEC were preincubated in the presence or absence of 1 mM neomycin for 15 min. The generation of prostacyclin and inositol phosphates in the presence or absence of 1 μM bradykinin (BK) was determined as described in Materials and Methods. Values are the means ± SD for three determinations.

* $P < 0.01$ compared to neomycin-free.

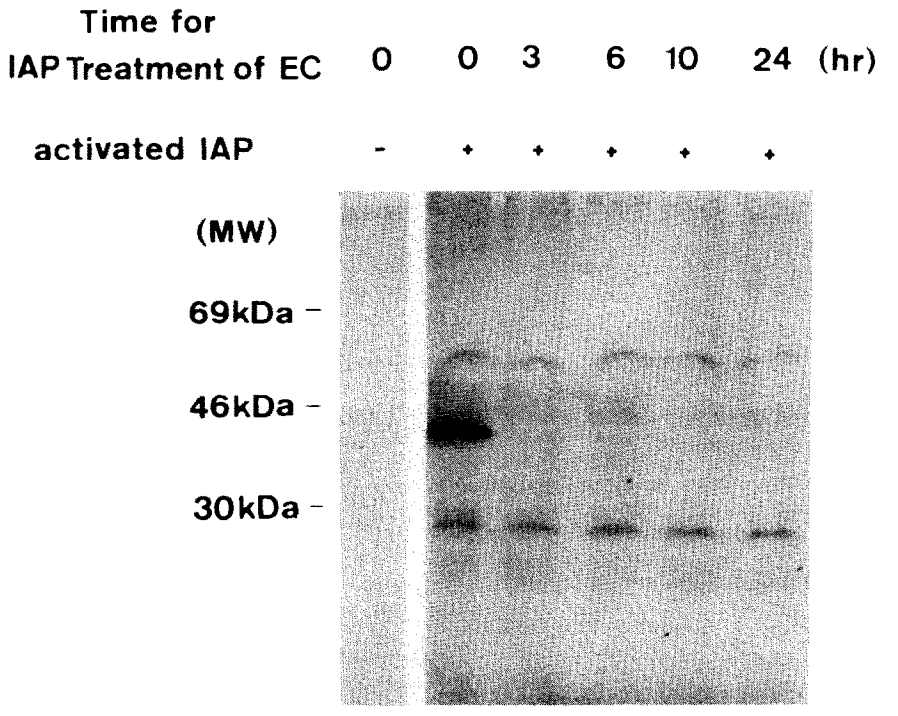

Fig. 3. [^{32}P]ADP-ribosylation of membranes prepared from PAEC preincubated with IAP. Confluent cells were treated with 100 ng/mL IAP for the indicated periods. The cells were washed and scraped with a rubber policeman in PBS containing 1 mM EDTA. [^{32}P]ADP-ribosylation in the cell membranes was measured as described in Materials and Methods.

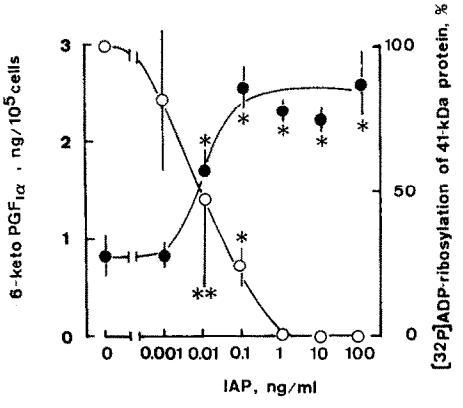

Fig. 4. Bradykinin-induced prostacyclin synthesis and ADP-ribosylation of the membrane protein with 41–42 kD. For prostacyclin synthesis, confluent PAEC in 12-well plates were cultured with various doses of IAP for 24 hr, washed, and then incubated with 1 μM bradykinin for 5 min. 6-keto $PGF_{1\alpha}$ in the medium was measured (●). For ADP-ribosylation, confluent cells in 175 cm^2 flasks were cultured with various doses of IAP for 24 hr. Membranes were prepared from PAEC and used for [^{32}P]ADP-ribosylation as described in Materials and Methods. The percent incorporation of the radioactivity is plotted against concentrations of the toxin used (○). Values are the means ± SD for three to four determinations. Key: (*) $P < 0.005$ and (**) $P < 0.05$ compared to IAP-free.

0.01 ng/mL, which was one order of magnitude lower than that in general use [10, 11] but close to that reported by Nishimoto *et al.* [31]. There was good correlation between the enhancement of bradykinin-induced prostacyclin synthesis and ADP-ribosylation of the membrane protein with M_r = 41–42 kD over the wide range of IAP concentrations. Figure 5 shows the effect of the removal of IAP from the incubation medium on the enhancement of bradykinin-induced prostacyclin synthesis. When the cells were washed extensively in PBS after being cultured with IAP for 3 hr and supplied to the additional culture in the IAP-free medium for 21 hr, the effect of IAP was unchanged.

Characterization of the IAP effect. Table 2 shows the effect of IAP on prostacyclin synthesis in response to several stimulants. IAP had no effect on the basal synthesis of prostacyclin, but stimulated prostacyclin synthesis evoked by bradykinin and vasopressin, both of which interact with the corresponding receptors, respectively, thus running the signal transduction systems to activate phospholipase A_2. On the other hand, the toxin did not enhance prostacyclin synthesis triggered by arachidonic acid, which is the substrate of prostaglandin G/H synthase, or by A23187, which raises the intracellular Ca^{2+} so that it can directly enhance phospholipase A_2 activity.

Effects of inhibitors of protein and mRNA syntheses on the IAP effect. Cycloheximide, an inhibitor of protein synthesis, concentration-dependently stimulated bradykinin-induced prostacyclin synthesis

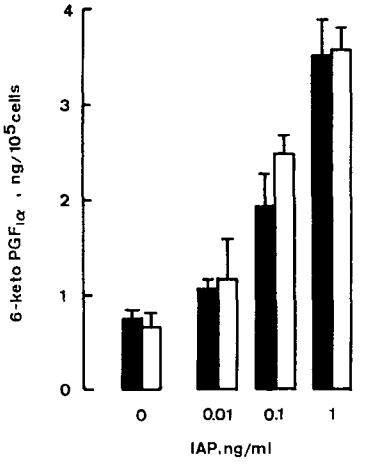

Fig. 5. Effect of IAP removal from the culture medium on enhancement of bradykinin-induced prostacyclin synthesis. Confluent PAEC were cultured for 3 hr in the absence or presence of IAP (0.01 to 1.0 ng/mL), then washed three times in PBS, and cultured continuously in IAP-free fresh medium for 21 hr (closed bars). As the control, another set of confluent cells were cultured with IAP for 24 hr (open bars). The cells were then washed and assayed for prostacyclin synthesis as described in Materials and Methods. Values are the means ± SD for three determinations.

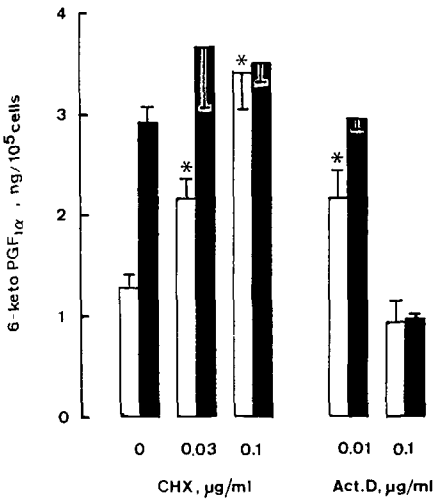

Fig. 6. Effect of cycloheximide (CHX) and actinomycin D (Act. D) on bradykinin-stimulated prostacyclin synthesis in IAP-treated and untreated PAEC. Confluent cells were cultured with various concentrations of CHX or Act. D in the absence (open bars) or presence (closed bars) of 100 ng/mL IAP. The cells were washed and then incubated with 1 μM bradykinin for 5 min. The media were assayed for 6-keto $PGF_{1\alpha}$. Values are the means ± SD for three determinations. Key: (*) $P < 0.005$ compared to inhibitor-free.

Table 2. Effect of IAP on prostacyclin synthesis in PAEC in response to several stimulants

Stimulant	6-keto $PGF_{1\alpha}$ (ng/well)	
	Control	IAP
None	0.18 ± 0.06	0.21 ± 0.03
Bradykinin (1 μM)	1.18 ± 0.30	3.52 ± 0.36*
Vasopressin (1 μM)	0.41 ± 0.08	0.83 ± 0.07*
A23187 (50 nM)	1.97 ± 0.21	1.48 ± 0.18
Arachidonic acid (1 μM)	1.36 ± 0.24	1.13 ± 0.05

PAEC were cultured at confluency for 24 hr with 100 ng/mL IAP. After the removal of the culture medium, they were washed and preincubated for 15 min in 0.5 mL of 199-Hepes and then incubated for 5 min with each stimulant. Values are the means ± SD for three to six determinations.

* $P < 0.005$ compared to control.

by itself, but had no effect in the presence of IAP (Fig. 6). Cycloheximide did not have any effect on the level of IAP substrate and its ADP-ribosylation (Fig. 7). At 0.01 μg/mL, actinomycin D, an inhibitor of RNA synthesis, also significantly ($P < 0.005$) stimulated bradykinin-induced prostacyclin synthesis without any effect on prostacyclin synthesis in IAP-treated cells. At 0.1 μg/mL, it decreased bradykinin-induced prostacyclin synthesis, and the same decrease was observed even when IAP was present (Fig. 6). In this case, cell viability (trypan blue exclusion) was 100%, but the shape of most cells was changed, indicating that the effect may be toxic.

Effect of IAP on lipocortin I content. Total proteins from PAEC untreated or treated with IAP were electrophoresed and immunoblotted with anti-rat lipocortin I antiserum. The immunoblot analysis of the untreated cells revealed a clear band with M_r = 38 kD, which showed the same mobility as mouse recombinant lipocortin I (Fig. 8B). The 38-kD band was not influenced by the exposure of the cells to 100 ng/mL IAP for 3 hr, but the exposure for 16 or 24 hr decreased the density of the band without decreasing amounts of total proteins detected in Coomassie blue staining (Fig. 8A).

Effect of IAP on the level of lipocortin I mRNA. Northern blot analysis was carried out to examine the influence of IAP on the expression of lipocortin I mRNA. As shown in Fig. 9, when the cells were exposed to 100 ng/mL IAP for 3, 16, and 24 hr, the content of lipocortin I mRNA, 1.3 kb, decreased by 30, 80, and 95% respectively. The content of lipocortin I mRNA in the cells treated with IAP was normalized by the content of β-actin mRNA.

DISCUSSION

In vascular endothelial cells, many agonists can alter phospholipid metabolism through two pathways: receptor-mediated activation of phospholipase A_2 and phospholipase C [1–6]. Phospholipase A_2 usually is described as having a requirement for Ca^{2+} [32], and its activation has been assumed to result from the elevation of intracellular Ca^{2+} induced by IP_3, which is generated by phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate [4–6]. However, Kaya *et al.* [7] very recently indicated that bradykinin stimulates the

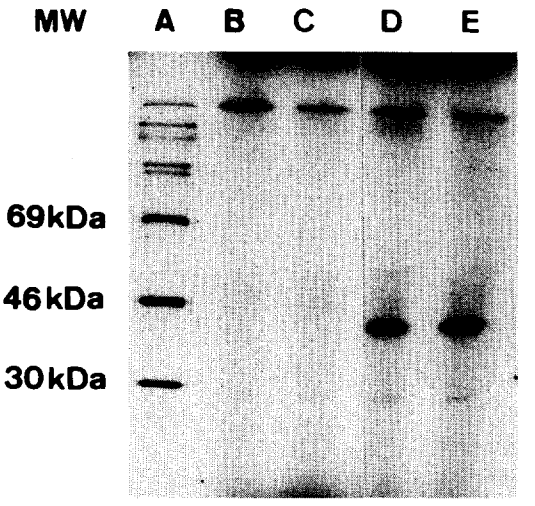

Fig. 7. Effect of cycloheximide (CHX) on ADP-ribosylation of the 41–42 kD membrane protein. Confluent cells in 175 cm^2 flasks were cultured with 100 ng/mL IAP (B, C) and/or 0.1 μg/mL CHX (C, E) for 24 hr. Lane D contained just the growth medium. Cells were washed and scraped with a rubber policeman in PBS containing 1 mM EDTA. [^{32}P]ADP-ribosylation of the proteins in cell membranes was measured as described in Materials and Methods. Lane A, marker proteins.

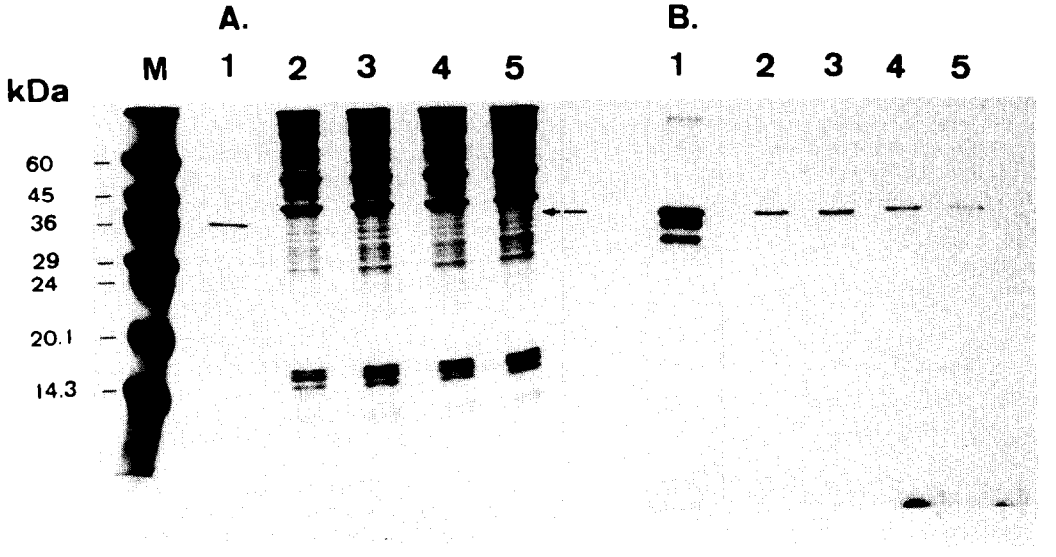

Fig. 8. Effect of IAP on the content of lipocortin I protein in PAEC. Total proteins from PAEC were separated on a polyacrylamide slab gel. (A) a section of the gel was stained with Coomassie blue; (B) another section was electrophoretically blotted and the blot was stained as described in Materials and Methods. Two bands with lower M_r values in lane 1 of Western blotting are thought to be highly immunoreactive breakdown products of mouse recombinant lipocortin I. Lane M, marker proteins; lane 1, mouse recombinant lipocortin I; lane 2, control PAEC; lanes 3, 4, and 5, PAEC cultured with 100 ng/mL IAP for 3, 16, and 24 hr, respectively.

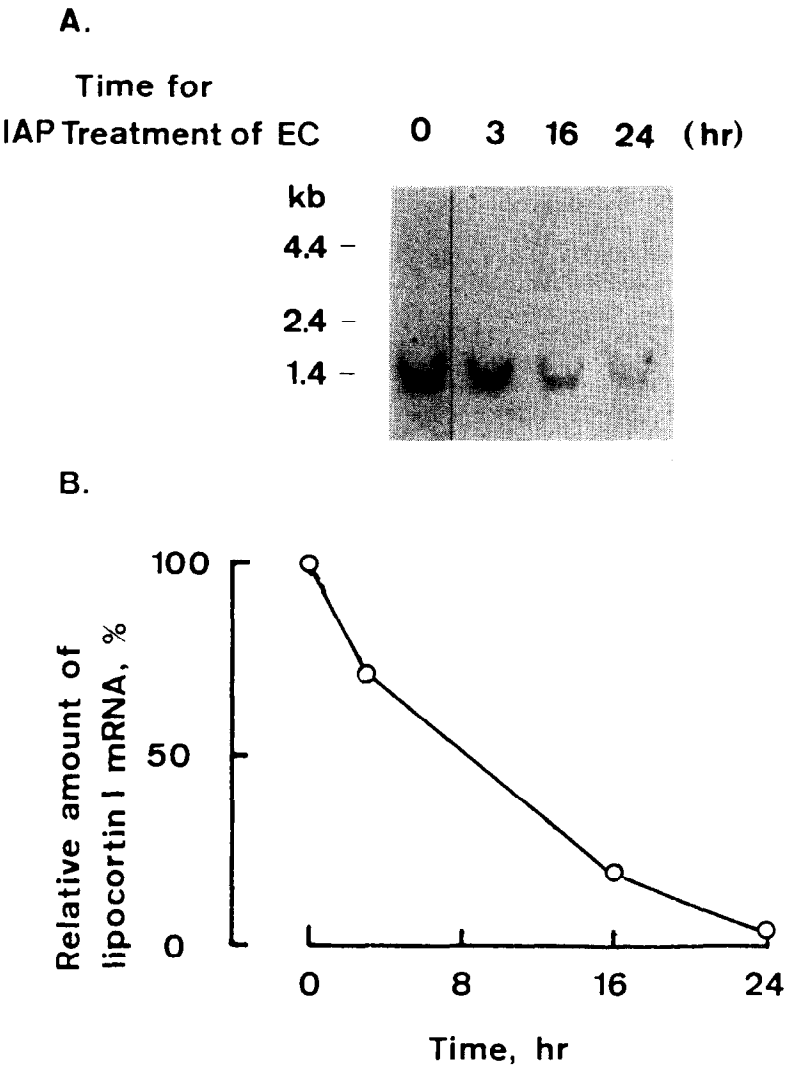

Fig. 9. Effect of IAP on the level of lipocortin I mRNA in PAEC. Confluent cells were cultured with 100 ng/mL IAP for the indicated periods. (A) Northern blot analysis of lipocortin I mRNA. Numbers at the left indicate the positions and lengths (kilobases) of RNA markers and hybridized RNA. (B) The content of lipocortin I mRNA normalized by the content of β-actin mRNA. The amounts of probe-specific transcripts were estimated by densitometry on a Joyce–Loebl model chromoscan 3 densitometer.

intracellular accumulation of unesterified arachidonic acid earlier than that of 1,2-diacylglycerol and phosphatidic acid in PAEC. Furthermore, we showed in this report that neomycin had no effect on prostacyclin production in PAEC under the condition where it inhibited IP_3 production, consistent with the result of Slivka and Insel [19] that neomycin selectively decreases phosphoinositide breakdown without decreasing prostaglandin E_2 synthesis in Madin–Darby canine kidney cells. These results suggest that the bradykinin-induced phospholipase A_2 activation pathway in PAEC is not required for phospholipase C-catalyzed IP_3 production. In addition to phospholipase A_2, a combined action of phosphatidylinositol- or phosphatidylcholine-specific phospholipase C and diglyceride lipase has been demonstrated as another pathway for arachidonate liberation [8]. It is possible that this pathway is operative in a part of prostacyclin synthesis in PAEC [1].

Neomycin did not block IP_1 and IP_2 accumulations. Low concentrations of neomycin have been reported to bind phosphatidylinositol 4,5-bisphosphate preferentially to phosphatidylinositol or phosphatidylinositol 4-phosphate [27, 33]. Such selective binding may inhibit phospholipase C cleavage of phosphatidyl 4,5-bisphosphate and release of IP_3, but not affect cleavage of other phosphoinositides, as described by Carney *et al.* [34]. Alternatively, neomycin may selectively stimulate the catabolism of IP_3 among inositol phosphates.

Recent studies have indicated that receptor-mediated activation of phospholipase A_2 involves G-protein [8–12]. In the present study we showed that the exposure of PAEC to IAP for 5 hr or longer leads to a significant increase in bradykinin-induced prostacyclin synthesis. Failure of IAP to enhance bradykinin-induced prostacyclin synthesis, observed by Clark *et al.* [13], may result from their

measurement of the synthesis at an inadequately early time point. Pirotton *et al.* [16] also demonstrated that IAP enhances the stimulating effect of ATP on prostacyclin release from bovine aortic endothelial cells, but they did not comment on the mechanism of IAP action. Burch *et al.* [17] demonstrated IAP-potentiated prostaglandin E_2 synthesis and suggested the possibility that an inhibitory G-protein may regulate phospholipase A_2 activity in a murine macrophage cell line, in addition to other possibilities. In PAEC, by contrast, the enhancing effect of IAP on bradykinin-induced prostacyclin synthesis was not due to the toxin-induced inactivation of the G-protein coupled to phospholipase A_2 because the enhancement of bradykinin-induced prostacyclin synthesis by IAP required another several hours after ADP-ribosylation of the IAP substrate with M_r = 41–42 kD (Figs. 3 and 4). The effect of IAP on prostacyclin synthesis seemed to be due to inactivation of a G-protein involved in the induction of lipocortin I or an endogenous inhibitor of phospholipase A_2 [35, 36], for the following reasons. First, although there was a time lag between ADP-ribosylation of the IAP substrate and the enhanced prostacyclin synthesis, the effect of IAP on prostacyclin synthesis was closely related to the preceding ADP-ribosylation, since there was good correlation between the enhancement of bradykinin-stimulated prostacyclin synthesis and ADP-ribosylation of the protein with M_r = 41–42 kD over a wide range of IAP concentrations. Further support was obtained by the fact that the removal of IAP from the medium at 3 hr did not cancel the enhancing effect of IAP on prostacyclin synthesis at 24 hr. Second, cycloheximide, like IAP, concentration-dependently enhanced bradykinin-stimulated prostacyclin synthesis, but had no effect in the presence of IAP. At 0.01 μg/mL, actinomycin D also showed a similar effect. These results suggest that the effect of IAP is similar to that of cycloheximide or actinomycin D. Third, the treatment of PAEC with IAP had no effect on arachidonic acid-induced prostacyclin synthesis, indicating that IAP acts on or before the step of arachidonic acid release. Fourth, IAP decreased the amount of the protein immunologically related to lipocortin I in parallel with the enhancement of prostacyclin synthesis. Fifth, IAP decreased the content of lipocortin I mRNA prior to the enhancement of prostacyclin synthesis. These results suggest that, since lipocortin I inhibits phospholipase A_2 [35, 36], the IAP-induced decrease in lipocortin I content may be involved in the enhancement of bradykinin-induced prostacyclin synthesis by IAP. At present, however, we cannot rule out the possibility that another inhibitor protein(s) than lipocortin I is involved in the IAP effect.

IAP is known to increase cellular cyclic AMP by inactivating G_i, which is involved in the inhibition of adenylate cyclase [29, 37]. Thus, the IAP-induced increase in cyclic AMP may give rise to activation of a protein kinase to phosphorylate and activate phospholipase A_2. However, this model does not reconcile with the present experiment because the enhancement of bradykinin-induced prostacyclin synthesis was not observed within 3 hr after the addition of IAP, when IAP influenced the cellular cyclic AMP level. Furthermore, when confluent PAEC were cultured with forskolin (10^{-7}–10^{-5} M) or dibutyryl cyclic AMP (10^{-6}–10^{-4} M) for 24 hr, both of which enhanced cellular cyclic AMP, prostacyclin synthesis was not influenced at all (data not shown).

The fact that IAP, which inactivates some G-protein(s) or transducer(s) in signal-transducing systems [10, 29, 30, 37], reduces the lipocortin I level suggests that some factor is involved in maintaining basal levels of lipocortin I. This factor may be derived from growth medium or may be an autocrine factor synthesized by PAEC.

The inhibitory protein of prostacyclin synthesis (possibly lipocortin I) seemed to act only on receptor-mediated activation of phospholipase A_2, because vasopressin, like bradykinin, but not calcium ionophore or arachidonic acid, produced more prostacyclin in the IAP-treated cells than in the untreated cells. Kannagi *et al.* [38, 39] proposed that some G-proteins belong to the membrane protein that can form and/or maintain "phospholipase A_2-susceptible domain" in membrane phospholipids in response to receptor-mediated stimulation. Lipocortins are known to have affinity to phospholipids to inhibit the activity of phospholipase A_2 [35, 36]. Together with our results, there is the possibility that lipocortin I binds phospholipids and blocks the G-protein-induced spreading of the phospholipase A_2-susceptible domain to suppress the release of arachidonic acid in response to receptor-mediated signals. Failure of IAP to enhance the Ca^{2+} ionophore A23187-induced prostacyclin synthesis may be because Ca^{2+} directly stimulates phospholipase A_2 without affecting the phospholipase A_2-susceptible domain. Alternatively, basal and A23187-stimulated prostacyclin productions may be due to the lipocortin-resistant population of phospholipase A_2 that is distinct from receptor-linked phospholipase A_2.

IAP-sensitive G-proteins have been reported to be involved in growth factor-triggered cell proliferation [30, 31], but little is known about the involvement of G-proteins in gene expression. Clark *et al.* [14] recently demonstrated that IAP inhibits rapid and transient tumor necrosis factor-induced induction of phospholipase A_2-activating protein. Here we showed that an IAP substrate seemed to be involved in the expression of lipocortin I, which may inhibit phospholipase A_2 activity in response to receptor-mediated signals. There are not only many agonists that are linked to signal-transducing G-proteins leading to direct activation of phospholipase A_2 [7–12], but there may be some agonists that are linked to G-proteins that indirectly regulate phospholipase A_2 activity through gene expression.

Lipocortin has been discovered as a glucocorticoid-inducible protein that inhibits phospholipase A_2 [35]. Recently, on the contrary, several investigators have demonstrated that glucocorticoid has no effect on the levels of mRNA and protein of lipocortin I [40–42]. However, they did not present any positive controls on the change of lipocortin I content. Since the content of lipocortin I in PAEC is actually altered by IAP, a breakthrough on this problem may

be made by examining the effect of glucocorticoid on the amount of lipocortin I in PAEC.

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