Calcitonin-Induced Increase in Phosphate Accumulation in LLC-PK₁ Cells Probably through Protein Kinase C Activation

YOSHIKAZU KINOSHITA, MASA AKI FUKASE, RUO HISHIKAWA AND TAKUO FUJITA

Third Division, Department of Medicine, Kobe University School of Medicine
7–5–1, Kusunoki-cho, Chuo-ku, Kobe 650, Japan

Abstract

To assess the role of protein kinase C and cAMP on the calcitonin-induced alteration of phosphate accumulation by renal tubular cells, the effects of phorbol esters, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), and DBcAMP on the phosphate accumulation in LLC-PK₁ cells were investigated. Calcitonin stimulated phosphate accumulation with a concomitant increase in cAMP production. Phorbol esters and 1-oleoyl-2-acetyl-glycerol, activators of protein kinase C, also stimulated the phosphate accumulation. Furthermore, H-7, an inhibitor of protein kinase C, inhibited a calcitonin-induced increase in phosphate accumulation significantly. Although DBcAMP by itself did not increase the phosphate accumulation, it enhanced the stimulatory effect of 12-0-tetradecanoyl phorbol-13-acetate on the phosphate accumulation. Accordingly, protein kinase C as well as cAMP might be involved in the calcitonin-induced increase in phosphate accumulation in LLC-PK₁ cells.

A number of studies have demonstrated a wide distribution of Ca²⁺-activated phospholipid dependent protein kinase (protein kinase C) in mammalian tissues, and the activation of this enzyme is implicated in transmembrane signaling for a variety of biologically active substances in the cells. We have recently reported the stimulatory effect of phorbol esters on phosphate accumulation in cultured renal tubular cells, and indicated that protein kinase C may possibly be involved in renal tubular phosphate accumulation (Kinoshita et al., 1986a).

However, at present, no information is available on the involvement of this enzyme in the hormonal control of renal phosphate handling.

LLC-PK₁ cells, derived from the porcine kidney, have been reported to possess calcitonin receptors and a Na⁺-dependent phosphate transport and are widely used as an experimental model for studying renal tubular functions (Rabito, 1983; Biber et al., 1983; Noronha-Blob et al., 1984; Caverzasio et al., 1985).

In the present study, using these cells, the role of protein kinase C in the calcitonin-induced alteration of phosphate accumulation was examined.
Materials and Methods

**Phosphate Accumulation Study**

LLC-PK₁ pig kidney cells (ATCC, CRL-1392) (Hull et al., 1976) were obtained from Flow Laboratories (Virginia) and were grown to a subconfluent state in 24 multiwell culture plates (2 cm²/well) (Corning, New York). These were prepared by seeding 1×10⁴ cells/well in 2 ml Dulbecco’s modification of Eagle’s minimum essential medium supplemented with 10% fetal calf serum (Grand Island Biological Co., New York). Phosphate accumulation measurement was performed according to the modified method of Biber et al (Biber et al., 1983) after 4 days’ culture. In brief, to study the effects of various substances on the phosphate accumulation in LLC-PK₁ cells, the cells were first incubated with 0.5 ml of media (modified Ham’s F12, Ca 1.2 mM, P 0.3 mM, PH 7.4) containing test agents or solvent as a control. When the effect of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) or N-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004) on the calcitonin-induced alteration of phosphate accumulation was investigated, the cells were preincubated with these inhibitors for 5 min before the addition of calcitonin. After 60 min incubation, the media were discarded and ³²P flux was initiated by adding 0.5 ml of media containing ³²P (modified Ham’s F12 (Ca 1.2 mM, P 0.3 mM, PH 7.4), ³²P 1 µCi/ml), followed by 5 min incubation. This incubation period was selected, since after 5 min of incubation, at which time the rate of accumulation was reported to be linear with time, the accumulation of phosphate was disclosed to be many times greater in the presence of Na⁺ than in the presence of choline⁺ in the medium (Noronha-Blob et al., 1984). ³²P accumulation was terminated by removing the medium and washing the cells four times with cold (4°C) normal saline. The cells were dispersed by trypsinization and ³²P accumulated in the cells was counted with a liquid scintillation counter.

**Effect of (Asu₁⁻⁷) ECT on cAMP Content**

The same culture conditions as in the phosphate accumulation study were used for this experiment. The effect of (Asu₁⁻⁷) eel calcitonin ((Asu₁⁻⁷) ECT) on cyclic adenosine 3’; 5’-monophosphate (cAMP) content in LLC-PK₁ cells was examined according to the method previously described (Kinoshita et al., 1985). In brief, the culture medium was removed and 0.5 ml of experimental medium (modified Ham’s F12 (Ca 1.2 mM, P 0.3 mM, PH 7.4)) was added to each well. The culture plates were continuously shaken for 60 min in a Dubnoff metabolic shaker at 37°C. Then (Asu₁⁻⁷) ECT solution was added to each well and they were incubated for 60 min. After incubation, the medium was removed and 1 ml of 6% trichloroacetic acid was immediately added to each well. Extraction and measurement of cAMP were performed according to our method previously reported (Kinoshita et al. 1986b).

**Reagents**

12-0-tetradecanoyl phorbol-13-acetate (TPA), 4α- and 4β-phorbol 12, 13-didecanoate, mezerein, and phorbol-12, 13-dibutyrate (PDBu) were purchased from Sigma Chemical Co. (St. Louis, MO). 1-oleoyl-2-acetyl-glycerol (OAG) was from Avanti Polar-Lipids, INC. (Birmingham, AL). H-7 and HA1004 were from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Dibutyryl cyclic adenosine 3’; 5’-monophosphate (DBcAMP) was kindly provided by Yamasa Shoyu Co. Ltd. (Choshi, Japan). All the calcitonin experiments were carried out with (Asu₁⁻⁷) ECT, which was a gift from Toyo Jozo Co. Ltd. (Shizuoka, Japan).

**Statistical Analysis**

The results were expressed as the mean ± S.E. of more than 4 different cultures. Significance of difference between groups was determined by Duncan’s new multiple range test (Duncan, 1955).

**Results**

**Effects of Calcitonin on Phosphate Accumulation and cAMP Production**

As shown in Fig. 1, the phosphate accumulation in LLC-PK₁ cells was stimulated by calcitonin dose-dependently from 1 to 1,000 ng/ml. Calcitonin, from 0.01 to 100 ng/ml, also increased the cAMP production dose-dependently and its effect reached a plateau at 100 ng/ml. From Fig. 1, the median effective concentration (EC50) of...
Fig. 1. Dose-dependency of (Asu¹⁷) ECT on the phosphate accumulation (●—●) and on the intracellular (○—○) and extracellular (×—×) cAMP content of LLC-PK₁ cells. Both phosphate accumulation and cAMP content were significantly increased by (Asu¹⁷) ECT. EC₅₀ for the phosphate accumulation (more than 10 ng/ml) was higher than that for the cAMP production (less than 0.1 ng/ml). Vertical lines represent the mean ± S. E. of more than 4 different cultures. * p < 0.01 significantly increased from the control.

calcitonin for the phosphate accumulation could be read as more than 10 ng/ml, and that for the cAMP production as less than 0.1 ng/ml.

Effects of DBcAMP, Phorbol Esters, and OAG on the Phosphate Accumulation

DBcAMP did not stimulate the phosphate accumulation at concentrations as high as 10⁻³ M (Fig. 2A). TPA, as low as 10 ng/ml, stimulated the phosphate accumulation significantly and dose-dependently increased it (Fig. 2B). 4α-phorbol 12, 13-didecanoate, a non-activator of protein kinase C, failed to augment the phosphate accumulation even at a concentration as high as 200 ng/ml, while 4β-phorbol 12, 13-didecanoate, an activator of protein kinase C, at the same concentration increased the accumulation significantly (Fig. 2C). In addition, PDBu, mezerein, and OAG, activators of protein kinase C (Kikkawa and Nishizuka, 1982; Nishizuka, 1984), increased the phosphate accumulation in LLC-PK₁ cells dose-dependently as indicated in Fig. 2D, E, F.

Effects of H-7 and HA1004 on a Calcitonin-Induced Increase in Phosphate Accumulation

The effects of H-7 and HA1004 on the calcitonin-stimulated phosphate accumulation are shown in Fig. 3. LLC-PK₁ cells were preincubated with H-7 or HA1004 for 5 min before stimulation with 1,000 ng/ml calcitonin. H-7 inhibited the calcitonin-stimulated phosphate accumulation significantly at 10 μM and caused 49% inhibition of the increased phosphate accumulation at 200 μM. On the other hand, HA1004 failed to inhibit the phosphate accumulation at 10 μM and inhibited it by only 17% at
Fig. 2. Dose-dependency of DBcAMP (A), TPA (B), 4α-phorbol 12, 13-didecanoate (○○○○) and 4β-phorbol 12, 13-didecanoate (●●●●) (C), PDBu (D), mezerein (E), and OAG (F) on the phosphate accumulation into LLC-PK₁ cells. TPA, 4α-phorbol 12, 13-didecanoate, PDBu, mezerein, and OAG increased the accumulation significantly, while DBcAMP and 4β-phorbol 12, 13-didecanoate failed to increase it. Vertical lines represent the mean ± S.E. of 8 different cultures. * P<0.01 significantly increased from the control.

200 μM. Neither H-7 nor HA1004 showed any inhibitory effect on the phosphate accumulation in the non-calcitonin-stimulated LLC-PK₁ cells.

**Combined Effects of DBcAMP and TPA on Phosphate Accumulation**

Although DBcAMP, at a concentration of 10⁻³ M, failed to augment the phosphate accumulation significantly (Fig. 2A), it enhanced the stimulatory effect of TPA on the phosphate accumulation when it was used in combination with 5, 10, 20, and 50 ng/ml TPA (Fig. 4).

**Discussion**

In the present series of experiments, the involvement of protein kinase C and cAMP on the calcitonin-induced increase in phosphate accumulation in LLC-PK₁ cells was demonstrated. Calcitonin is reported to increase cAMP production by the renal tubules and considered to transduce its signal through cAMP generation (Marx et al., 1972; Loreau et al., 1975). Also in our cells, calcitonin stimulated the phosphate accumulation with a concomitant increase in cAMP production. However, EC₅₀ of calcitonin for the cAMP production and for the phosphate accumu-
Fig. 3. Dose-dependent effects of H-7 and HA1004 on the (Asu\textsuperscript{1,7}) ECT-stimulated phosphate accumulation. When incubated with 1,000 ng/ml (Asu\textsuperscript{1,7}) ECT for 60 min, the phosphate accumulation into the cells was increased to \( (97.2 \pm 2.3) \times 10^{-12} \text{M/10}^5 \text{cells/5 min} \) from the basal value, \( (31.3 \pm 1.3) \times 10^{-12} \text{M/10}^5 \text{cells/5 min} \). When LLC-PK\(_1\) cells were preincubated for 5 min with H-7 before (Asu\textsuperscript{1,7}) ECT stimulation, the (Asu\textsuperscript{1,7}) ECT-stimulated phosphate accumulation was remarkably inhibited. HA1004, on the other hand, inhibited the (Asu\textsuperscript{1,7}) ECT-stimulated phosphate accumulation only slightly. Vertical lines represent the mean \( \pm \) S.E. of 8 different cultures.

* P<0.01 significantly different from the value obtained in the absence of H-7 or HA1004.

Fig. 4. Combined effects of DBcAMP and TPA on the phosphate accumulation. The effect of TPA was synergistically enhanced by 10\textsuperscript{-3} M DBcAMP. Vertical lines represent the mean \( \pm \) S.E. of 8 different cultures.

* P<0.01 significantly different from the value obtained in the absence of DBcAMP.

(●—●): without DBcAMP.
(○—○): with 10\textsuperscript{-3} M DBcAMP.

...be explained solely by cAMP production. Indeed, DBcAMP by itself did not stimulate the phosphate accumulation.

On the other hand, our experiments with TPA, 4\textbeta- phorbol 12, 13-didecanoate, PDBu, mezerein, and OAG, activators of protein kinase C, strongly suggested the involvement of protein kinase C in the stimulation of phosphate accumulation in LLC-PK\(_1\) cells. H-7 and HA1004, newly synthesized compounds related to isoquinolinesulfonamides, have different Ki values for protein kinase C (Inagaki \textit{et al.}, 1984; Kawamoto and Hidaka, 1984; Hidaka...
H-7 is known to be a potent inhibitor of protein kinase C (Ki value is 6.0 μM), whereas HA1004 is a weak inhibitor of this enzyme (Ki value is 40.0 μM) (Hidaka et al., 1984). In our experiments, H-7 inhibited the calcitonin-stimulated phosphate accumulation significantly, while HA1004 inhibited it to a lesser degree. Since these two inhibitors have similar Ki values for cAMP dependent protein kinase, the difference in their effect on phosphate accumulation might be attributable to their different Ki values for protein kinase C and suggests the possible involvement of protein kinase C in the calcitonin-stimulated phosphate accumulation in LLC-PK₁ cells. Although additional work will be necessary to obtain direct evidence of calcitonin-stimulated protein kinase C activation, our data showing that calcitonin stimulates phosphatidylinositol turnover in LLC-PK₁ cells (Kinoshita et al., 1987) also support the present results.

While DBcAMP did not stimulate the phosphate accumulation by itself, it enhanced the TPA-induced increase in phosphate accumulation. These results show that the calcitonin-stimulated increase in phosphate accumulation in LLC-PK₁ cells is mediated through at least two signal transduction systems. That is, one of them might be through the activation of protein kinase C probably by diacylglycerol generated as a result of phosphatidylinositol turnover, and the other through the cAMP production which augments the effect of the former system. Although additional studies might be necessary to investigate whether calcitonin alters the renal tubular phosphate transport via the same mechanism, our investigation on LLC-PK₁ cells might help to disclose the intracellular mechanism of calcitonin action.

References


Kinoshita, Y., M. Fukase, T. Yamatani, R. Hishikawa and T. Fujita (1986a). Phorbol esters stimulate phosphate accumulation syn-


