Role of Phosphodiesterase 4 Isoenzyme in Alkaline Phosphatase Activation by Calcitonin in Porcine Kidney LLC-PK$_1$ Cells

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ABSTRACT—To confirm the intracellular signal transduction in regulation of alkaline phosphatase (ALP) activity by calcitonin in kidney tubular cells, effects of several inhibitors of cyclic nucleotide phosphodiesterase (PDE) isoenzymes and cyclic AMP-dependent protein kinase (PKA) on the action of salmon calcitonin in porcine kidney tubular epithelial cells LLC-PK$_1$ were examined. A confluent culture of LLC-PK$_1$ cells was treated with calcitonin and inhibitors in Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin, and intracellular cyclic AMP content and ALP activity were measured after incubation for 30 min and 48 hr, respectively. Calcitonin and PDE 4 inhibitors increased cyclic AMP level and ALP activity in the cells, and PDE 4 inhibitors synergistically potentiated the effects of calcitonin. Calcitonin induced ALP activation by treatment for the first 1 hr, as well as continuous treatment for 48 hr, while it never increased the enzyme activity just after 1-hr exposure. Rolipram, an inhibitor of PDE 4 isoenzyme, induced ALP activation by itself and in combination with calcitonin by only a long term treatment (48 hr). The activation of ALP by calcitonin and rolipram each alone and in combination was completely abolished by a PKA inhibitor, H-89. These results confirm that calcitonin induces ALP activation through the cyclic AMP-PKA pathway and that PDE 4 isoenzyme is closely associated with the calcitonin-receptor system and plays a major role in hydrolysis of cyclic AMP produced in the kidney tubular cells.

Keywords: Calcitonin, Alkaline phosphatase, Phosphodiesterase 4, Kidney, LLC-PK$_1$ cell

Calcitonin is a hormone, which lowers serum Ca^{2+} by stimulating urinary excretion (1, 2) and inhibiting osteoclastic bone resorption (3). The initial step of the action of calcitonin is binding to the membrane receptors of the cells, linking to the G proteins associated with adenylate cyclase and phospholipase C pathways (4-8). On the other hand, although alkaline phosphatase (ALP) plays an important role in renal tubular functions including Ca^{2+} and phosphorus ion excretion (9, 10), there is little information about the regulation of the enzyme activity by calcitonin.

This study deals with cyclic AMP-mediated calcitonin action using several inhibitors of cyclic nucleotide phosphodiesterase (PDE) isoenzymes and cyclic AMP-dependent protein kinase (PKA) in porcine kidney tubular epithelial cells LLC-PK$_1$.

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MATERIALS AND METHODS

Agents

Salmon calcitonin (Bachem Feinchemikalien AG, Bubendorf, Switzerland) was dissolved in 1% sodium acetate containing 0.1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA) and used after at least 100-fold dilution with culture medium.

3-Isobutyl-1-methylxanthine (IBMX), amrinone and zaprinast were purchased from Sigma. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) were from Seikagaku Kogyo Co., Tokyo. 1-*n*-Butyl-3-*n*-propylxanthine (XT-44) (11), denbufylline (12) and rolipram (13) were synthesized in our laboratory, according to the reported

methods. These agents were dissolved in dimethylsulfoxide and used after 200-fold dilution with culture medium.

Cell culture and treatment

The porcine kidney epithelial cell line LLC-PK_1 was originally purchased from Flow Laboratories, Virginia, USA. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37℃ in a humidified 5% CO_2 incubator; and at confluence, the cells were dispersed with 0.25% trypsin-EDTA at 37℃ for 10 min and passaged to 35-mm plastic dishes at a density of 1.5×10^5 cells/2 ml/ dish. At subconfluence, the culture medium was removed, the cells were rinsed twice with phosphate-buffered saline (pH 7.4) and incubated in DMEM supplemented with 0.1% BSA for about 24 hr, by which time they had become confluent, and then used.

Cyclic AMP assay

The confluent LLC-PK_1 cell layers were treated with test agents for 30 min, and cells were collected and homogenized in cold ethanol. The cell-ethanol extract was dried in nitrogen stream and dissolved in the assay buffer. Cyclic AMP content was measured using a cyclic AMP enzyme immunoassay kit (Amersham International plc, Buckinghamshire, UK) according to the manufacturer's protocol.

ALP assay

The confluent culture was treated with test agents for the first 1 hr, the last 1 hr, or continuously during incubation for 48 hr. At the end of incubation, the cells were suspended in 0.2% Nonidet P-40 and sonicated in an ice bath; after centrifugation, ALP activity in the supernatant was measured by the Bessey-Lowry method using *p*-nitrophenylphosphate as a substrate. The enzyme activity was expressed in nmol/(min·mg protein).

Protein assay

Protein content in a part of cell lysate was measured by the Lowry-Folin method using BSA as a standard.

Statistical analyses

Values are expressed as the means±S.E.M. of three to five independent experiments. Statistical significance was measured with Student's *t*-test and Duncan's test.

RESULTS

Salmon calcitonin increased the intracellular cyclic AMP in a concentration-dependent manner up to 0.1 nM, activating the ALP activity about twofold, but at higher concentrations, the enzyme activity reached a plateau or decreased in LLC-PK_1 cells (Fig. 1). Figure 2 shows the effects of PDE inhibitors (each 10 μM) on the ALP activity. A non-selective PDE inhibitor, IBMX, increased the ALP activity, and PDE 4-selective inhibitors, rolipram (13), XT-44 (14, 15) and denbufylline (16), acti-

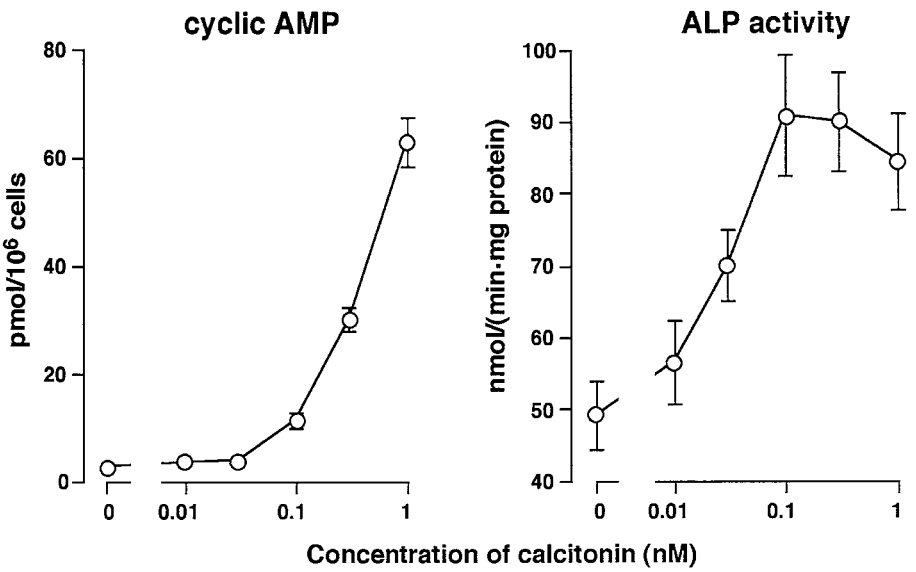

Fig. 1. Effects of salmon calcitonin on the cyclic AMP content and ALP activity in LLC-PK_1 cells. When salmon calcitonin was added to the confluent cell culture, intracellular cyclic AMP content was measured after treatment for 30 min and ALP activity in the cells was measured after treatment for 48 hr. Data are the means±S.E.M.

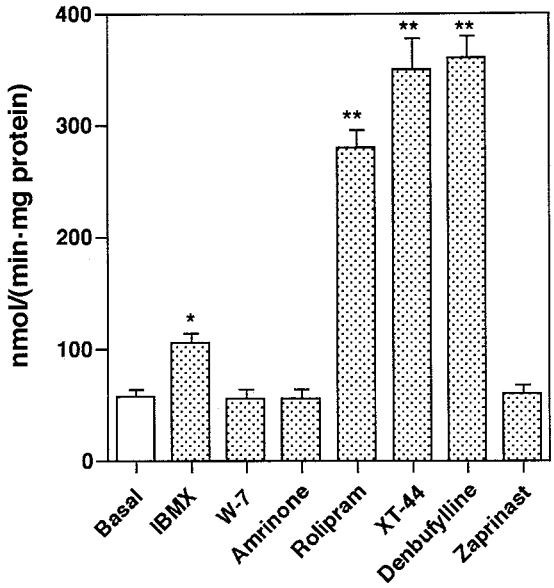

Fig. 2. Effects of PDE inhibitors on ALP activity in LLC-PK$_1$ cells. ALP activity was measured after treatment with an agent, each at 10 μM, for 48 hr. Data are the means±S.E.M. *, **Significantly different from the basal activity at $P<0.05$ and $P<0.01$, respectively.

vated the enzyme activity over fivefold of the untreated activity. The calmodulin inhibitor W-7 (17), the PDE 3 inhibitor amrinone (18), and the PDE 5 inhibitor zaprinast (19) hardly affected the enzyme activity. Although rolipram itself only slightly increased the intracellular cyclic AMP content after treatment for 30 min, in combination with 0.1 nM calcitonin, it significantly elevated the cyclic AMP content; similarly for ALP activity, the PDE 4 inhibitor also synergistically increased the effect of calcitonin (Fig. 3). Inhibitors of PDE 3 and 5 isoenzymes influenced neither the cyclic AMP content nor the ALP activity induced by calcitonin (Fig. 4). W-7 was also ineffective on the calcitonin actions.

On the other hand, as shown in Fig. 5, calcitonin (0.1 nM) increased the ALP activity to about twofold the basal level by a 1-hr pulse exposure and 47-hr incubation, as well as continuous treatment for 48 hr, but not by only 1-hr treatment. One of the PDE 4 inhibitors, rolipram (1 μM), hardly influenced the ALP activity by short term treatment (1 hr), both before and after incubation, and needed a long term treatment (48 hr) to significant increase the enzyme activity. The combined treatment with calcitonin and rolipram for 48 hr produced extremely high ALP activity. The ALP activity induced by both calcitonin and rolipram was completely inhibited by the PKA inhibitor H-89 (20) (Table 1).

DISCUSSION

Cyclic AMP is hydrolyzed and inactivated by PDE in the cells, and several tissues have been reported to contain seven families of PDE isoenzymes (21, 22). This study using the porcine kidney tubular epithelial cell line LLC-PK$_1$ indicated that among the PDE inhibitors, only PDE 4-selective inhibitors such as rolipram (13), XT-44 (14, 15) and denbufylline (16) not only increased ALP activ-

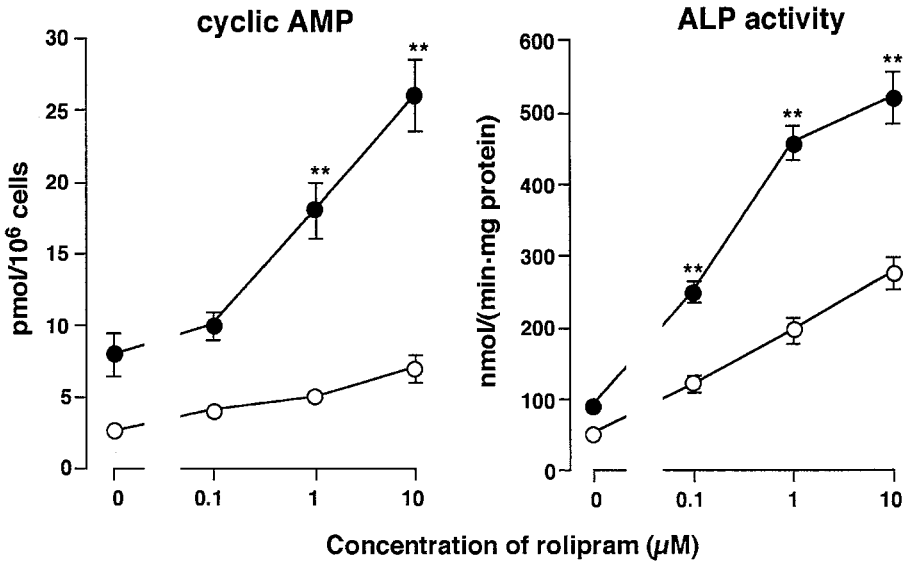

Fig. 3. Effects of rolipram on the cyclic AMP content and ALP activity induced by salmon calcitonin in LLC-PK$_1$ cells. Cells were treated with varying concentrations of rolipram in the absence (○) or presence of 0.1 nM salmon calcitonin (●) for 48 hr. Data are the means±S.E.M. **Significantly different from both activities treated with calcitonin and rolipram, each alone, at $P<0.01$.

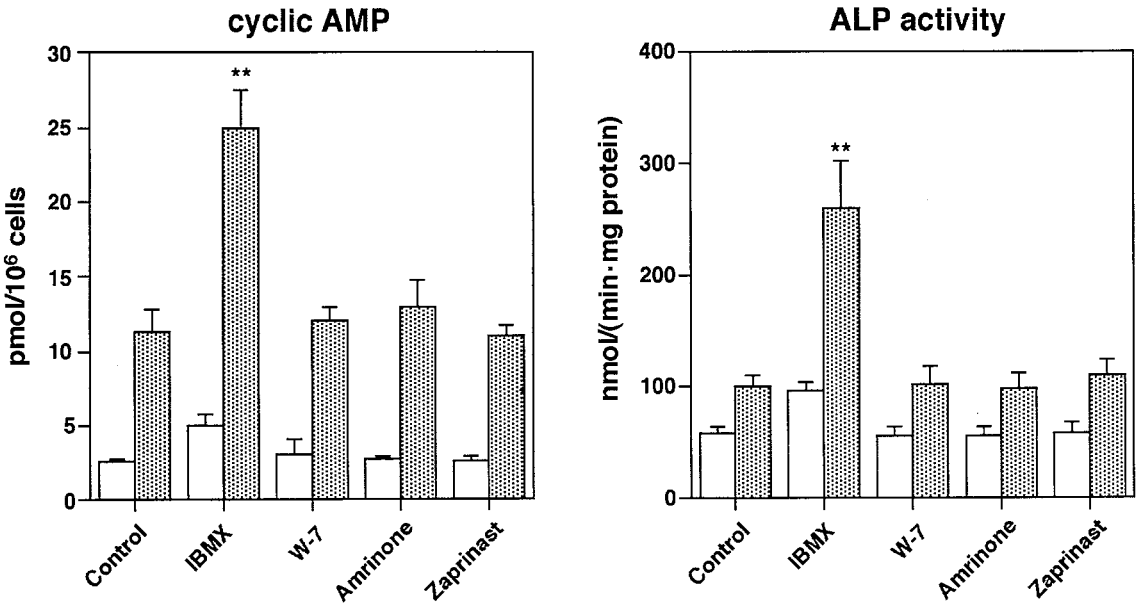

Fig. 4. Effects of three PDE inhibitors and W-7 on the cyclic AMP content and ALP activity induced by salmon calcitonin in LLC-PK$_1$ cells. Cells were treated with a PDE inhibitor, each at 10 μM, in the absence (open column) or presence of 0.1 nM salmon calcitonin (dotted column) for 48 hr. Data are the means±S.E.M. **Significantly different from the activity treated with calcitonin alone at $P<0.01$.

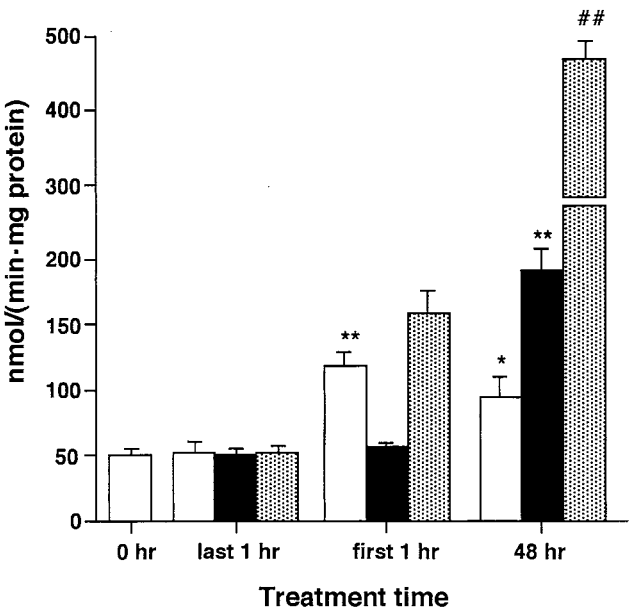

Fig. 5. Effects of salmon calcitonin (0.1 nM) and rolipram (1 μM) on ALP activity in LLC-PK$_1$ cells. ALP activity was measured after incubation of confluent cell culture for 48 hr, which was exposed to calcitonin (open column), rolipram (closed column) or both agents (dotted column) for the last 1 hr, the first 1 hr or the full 48 hr. Data are the means±S.E.M. *, **Significantly different from the basal activity at $P<0.05$ and $P<0.01$, respectively. ##Significantly different from both activities by calcitonin and rolipram, each alone, at $P<0.01$.

Table 1. Effect of H-89 on the ALP activity induced by calcitonin and rolipram

	ALP activity [nmol/(min·mg protein)]	
	None	H-89 (20 μM)
Basal	56.3±5.2	52.3±3.4
Rolipram (1.0 μM)	142±11	56.7±6.1**
Calcitonin (1.0 nM)	177±6	61.6±5.9**
Calcitonin + Rolipram	361±15	67.4±3.6**

Data are the means±S.E.M. from three to five experiments. **Significantly different from the values in the absence of H-89 at $P<0.01$.

ity by themselves but also synergistically potentiated the effects of calcitonin in cyclic AMP accumulation and ALP activation in the cells (Figs. 2 and 3). The inhibitors of PDE 3 and PDE 5 and the calmodulin inhibitor W-7 (17) did not influence the cyclic AMP content and ALP activity in the cells and effects of calcitonin (Figs. 2 and 4). Tomkinson et al. (23) and we (14) reported that the PDE 4 isoenzyme is functionally associated with β_2-adrenoceptors in guinea pig tracheal muscle and inhibition of this enzyme potentiates the ability of β_2-stimulants to increase the intracellular cyclic AMP content, whereas tracheal muscle has multiple PDE isoenzymes, at least PDEs 1 to 5. The present study also suggests in LLC-PK$_1$ cells that the PDE 4 isoenzyme is closely associated with the calcitonin receptor-adenylate cyclase system and

dominantly hydrolyzes cyclic AMP produced after the receptor stimulation. Other PDE isoenzymes including calmodulin-dependent PDEs may not be located near the receptor system or may not be present in the kidney cells. It is evident that the cyclic GMP pathway is not related to the ALP activity in the cells because zaprinast, which is an inhibitor of cyclic GMP-specific PDE (PDE 5) (19), did not show any effect on the basal activity and the calcitonin-stimulated activity.

On the other hand, calcitonin induced ALP activation by a 1-hr pulse treatment, as well as continuous treatment for 48 hr, while it never increased the enzyme activity just after treatment for 1 hr (Fig. 5). This suggests that the increase in the ALP activity by calcitonin is accompanied by the gene expression or protein synthesis. Rolipram induced ALP activation by itself and in combination with calcitonin only after a long term treatment (Fig. 5). This seems that rolipram may need a long time to accumulate a sufficient amount of cyclic AMP to exhibit biological activity. The ALP activity increased by both rolipram and calcitonin was completely abolished by an inhibitor of PKA, H-89 (20), which did not influence the intracellular cyclic AMP level (data not shown). This indicates that these agents activate the enzyme through the cyclic AMP-PKA cascade.

Moreover, a 48-hr treatment with calcitonin up to a concentration of 0.1 nM only increased the ALP activity about twofold, which was similar or lower than that by a pulse-treatment for 1 hr and incubation for 47 hr (Figs. 1 and 5), while the PDE 4 inhibitor rolipram increased the enzyme activity by about four- to five-fold in a concentration-dependent manner (Fig. 3). This phenomenon may indicate the homologous down-regulation or desensitization of calcitonin receptor. PKA-mediated internalization (24) and decrease in mRNA expression of calcitonin receptor (25) have been reported. Desensitization by receptor phosphorylation by PKA or receptor-specific protein kinase may also be considered, as reported in β_2-adrenoceptors (26, 27).

In conclusion, calcitonin induces ALP activation through the cyclic AMP and the PKA pathway, and the PDE 4 isoenzyme is closely associated with the calcitonin-receptor system and plays a major role in hydrolysis of cyclic AMP produced in kidney tubular cells.

Acknowledgments

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