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Stimulatory Effect of Endothelin-1 on Na-Dependent Phosphate Transport and Its Signaling Mechanism in Osteoblast-Like Cells

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Abstract Endothelin-1 (ET-1) has been reported to modulate bone metabolism both in vivo and in vitro. In the present study, we investigated the effect of ET-1 on inorganic phosphate (Pi) transport in osteoblast-like cells, which is now considered to be important for the initiation of bone matrix calcification. ET-1 time- and dose-dependently stimulated Na-dependent Pi transport in mouse calvaria-derived osteoblast-like MC3T3-E1 cells, and this effect was dependent on transcriptional and translational process. Kinetic analysis indicated that the change in Pi transport activity induced by ET-1 was due to alteration in the number of the Pi transporter. BQ123, a selective antagonist for ET_A receptor, suppressed the ET-1-induced Pi transport, but BQ788, a selective antagonist for ET_B receptor, had no effect. The inhibition of phosphoinositide hydrolysis by phospholipase C (PLC) partially attenuated the Pi transport by ET-1. Propranolol, which inhibits phosphatidic acid phosphohydrolase, also suppressed ET-1-induced Pi transport. On the contrary, indomethacin did not affect the stimulatory effect of Pi transport by ET-1. Calphostin C, a protein kinase C (PKC) inhibitor, significantly blunted the stimulatory effect of ET-1 on Pi transport. Combined effect of PMA and ET-1 on Pi transport was not additive. Pi transport induced by ET-1 was also suppressed in PKC down-regulated cells. In conclusion, the results of the present study indicate that, in MC3T3-E1 osteoblast-like cells, ET-1 acting through ET_A receptor links to a stimulation of Pi transport via activation of PKC through both phosphoinositide and phosphatidylcholine hydrolyses. J. Cell. Biochem. 83: 47–55, 2001.

Key words: endothelin-1; ET_A receptor; phospholipase C and D; protein kinase C

Endothelins (ET) are a family of 21-residue peptides synthesized by a selected endothelial and epithelial cells that act in a paracrine fashion, and are now known to have three isotypes, ET-1, ET-2, and ET-3 [Yanagisawa et al., 1988; Simonson and Dunn, 1990; Masaki, 1993]. In addition to potent vasoconstrictive and vasopressor activities, widespread expression of ET and their biological actions have been reported in various tissues [Simonson and Dunn, 1990; Masaki, 1993]. ET binds to three types of receptors denoted ET_A, ET_B, and ET_C [Pollock et al., 1995]. ET_A receptors are selective for ET-1 and ET-2, whereas ET_B receptors bind to ET-1, ET-2, and ET-3 with equal potency. ET_C has also been identified, but its physiological significance is uncertain.

In bone, it is widely believed that the vasculature plays an important role in bone remodeling under normal and pathological conditions. The effects of ETs on bone tissue and bone cells have been reported by several groups [Zaidi et al., 1993; Stern et al., 1995]. In bone organ culture, ET has been reported to stimulate bone resorption and anabolism such as collagen and noncollagen protein synthesis [Tatrai et al., 1992]. In osteoblasts, it has been shown that ET_A and ET_B receptors exist on osteoblasts [Nambi et al., 1995; Hiruma et al., 1998], and that ET-1 stimulates DNA synthesis and reduces alkaline phosphatase activity [Takuwa et al., 1990]. As for intracellular signaling system, it has been reported that ET-1 stimulates Ca mobilization, phosphoinositide (PI) hydrolysis by phospholipase C (PLC), phosphatidylcholine (PC) hydrolysis by phospholipase D (PLD), arachidonic acid release and p42/44 and p38 mitogen activated-protein

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(MAP) kinases activation in osteoblast-like cells [Takuwa et al., 1989, 1990; Suzuki et al., 1994, 1997; Semler et al., 1995; Kozawa et al., 1997; Kawamura et al., 1999a,b]. ET-1 affects intracellular signaling mainly through its binding to ET_A receptor in osteoblast-like MC3T3-E1 cells [Suzuki et al., 1997; Hiruma et al., 1998; Matsuno et al., 1998], but ET_B receptors have been also reported to be involved in ET-1-induced PLC activation and calcium transient in osteoblastic osteosarcoma cells [Nambi et al., 1995]. Although, ETs have recently been reported to inhibit the mineralization of osteoblastic cells in vitro [Hiruma et al., 1998], there is another report saying that ET-1 stimulates both proliferation and the formation of bone nodules in osteoblastic progenitor cells [von Schroeder et al., 2000]. So, the exact role of ET on bone formation and mineralization is still unknown.

Inorganic phosphate (Pi) is an essential nutrient in the process of energy metabolism in ubiquitous cells [Takeda et al., 2000]. In skeletal tissues, Pi is an essential element for the physiological functioning of osteogenic cells, not only because it is an integral component of apatite crystal but also because it can affect the production rate of the bone matrix [Caverzasio et al., 1996]. The functioning role of Pi transport in osteogenic cells for bone mineralization has been considered [Caverzasio et al., 1996], because Pi transport in the matrix vesicles, which are exracellular organelles formed by budding of the plasma membrane of osteogenic cells, was found to be critical for the development of matrix vesicle calcification [Montessuit et al., 1991]. In addition, the characterization of a Pi transport system in matrix vesicles has been reported to be similar to those of the Pi transport system present in the plasma membrane of bone-forming cells, such as osteoblasts [Montessuit et al., 1995]. Several hormones and growth factors have been reported to stimulate Na-dependent Pi transport in osteoblastic cells through protein kinase C (PKC)-dependent or -independent pathway [Selz et al., 1989; Imai et al., 1996; Zhen et al., 1997; Veldman et al., 1998; Suzuki et al., 2000].

In the present study, we investigated the effect of ET-1 on Pi transport system in osteoblast-like cells, and found that ET-1 acting through ET_A receptor links to a stimulation of Pi transport via activation of PKC through both PI hydrolysis by PLC and PC hydrolysis by PLD in osteoblast-like cells.

MATERIAL AND METHODS

Chemicals

Cell culture reagents were purchased from Flow Laboratories (ICN Biochemicals, Inc., Costa Mesa, CA) and fetal calf serum (FCS) was from Gibco (Life Technologies Ltd., Paisley, UK). ET-1 was purchased from Peptide Institute, Inc. (Minoh, Japan). Cyclo-^{D}-Trp-^{D}-Asp-Pro-^{D}-Val-Leu (BQ123) and *N-cis*-2,6-di-methylpiperidinocarbonyl-^{D}-γMeLeu-^{D}-Trp(COOMe)-^{D}-Nle-ONa (BQ788) were kindly provided by Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan). U73122 was from Cayman Chemical (Ann Arbor, MI). U73343, propranolol hydrochloride (propranolol), indomethacin and 12-*O*-tetradecanoyl phorbol-13-acetate (PMA) were from Sigma Chemical Company (St Louis, MO). Calphostin C, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) and 4-(4-flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazone (SB203580) were from Calbiochem-Novabiochem (La Jolla, CA). $H_3[^{32}P]O_4$ and L-[3-^{3}H]-alanine were from DuPont de Nemours (Brussel, Belgium). All other chemicals were from standard laboratory suppliers and were of the highest purity available.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvariae were generously provided by Dr. J. Caverzasio (University Hospital of Geneva, Geneva, Switzerland). Cells were seeded into 24-well plates (40×10^3 cells/well) and reached confluence after 4 days. Cultures were maintained in α-MEM containing 10% FCS at 37°C in a humidified atmosphere of 5% CO_2-95% air and medium was changed every 2–3 days.

Influence of ET-1 on Pi Transport Activity

The effects of ET-1 on Pi transport was analyzed in confluent MC3T3-E1 cells cultured in α-MEM containing 2% FCS for 24 h. Then, they were incubated for 6 h in the same culture medium with either various doses of ET-1 or its vehicle. Following cell treatment with agents, Pi transport activity was determined in Earle's buffered salt solution (EBSS) containing 0.05–2.5 mM labeled $H_3[^{32}P]O_4$ or 0.1 mM [^{3}H]alanine. Before the transport assay, the cell layer was rinsed three times with EBSS without radioactive or cold substrate. The transport

measurement started after adding 0.3 ml of EBSS containing the labeled substrate (1 μCi/ml). After 6 min incubation for Pi or 2 min for alanine transports analysis, which corresponds to time points representing respective initial rate of transport activities in these cells, the uptake solution was aspirated and the cell layer was rinsed three times with 0.3 ml of ice-cold (4°C) substrate free EBSS. The cells were then solubilized with 0.25 ml of 0.2 N sodium hydroxide and the radioactivity contained in a 200 μl aliquot was counted by a standard liquid scintillation technique (Beckman LS-6000IC). As previously documented, preliminary experiments indicated that the Na-independent component of Pi transport in these cells was less than 10% of the total uptake of Pi determined in presence of 143 mM sodium chloride and this component was not influenced by ET-1 (data not shown). Therefore, unless specified, this Na-independent component was neglected.

Statistical Analysis

Results are expressed as mean±SEM. A two-sided unpaired Student's *t*-test or ANOVA for multiple comparisons was used for statistical analysis. A difference between experimental groups was considered to be significant when the *P* value was $< 5\%$.

RESULTS

Characteristics of Pi Transport Stimulation by ET-1 in MC3T3-E1 Osteoblast-Like Cells

In MC3T3-E1 cells, the exposure to various doses of ET-1 for 6 h showed dose-dependent increase in Pi transport (Fig. 1). The stimulatory effect was detected with 10 pM of ET-1 and maximal stimulation was reached at the dose of 1 nM. The effect was time-dependent and a small but significant effect was detected after only 15 min of incubation (Fig. 2). A maximal response was reached after 6 h and maintained for at least 24 h. Although, ET-1 has been reported to stimulate DNA synthesis of osteoblast-like [Takuwa et al., 1989, 1990], DNA content of the cells stimulated by 10 nM ET-1 for 6 h was not changed in comparison with that of control cells (data not shown). ET-1 (10 nM for 6 h) showed little effect on the alanine transport system, which is also driven by the transmembrane gradient of Na (vehicle, 102.4±2.4 pmol/2 min/well; ET-1, 100.8±8.1 pmol/2 min/well). Kinetic analysis indicated that this effect of ET-

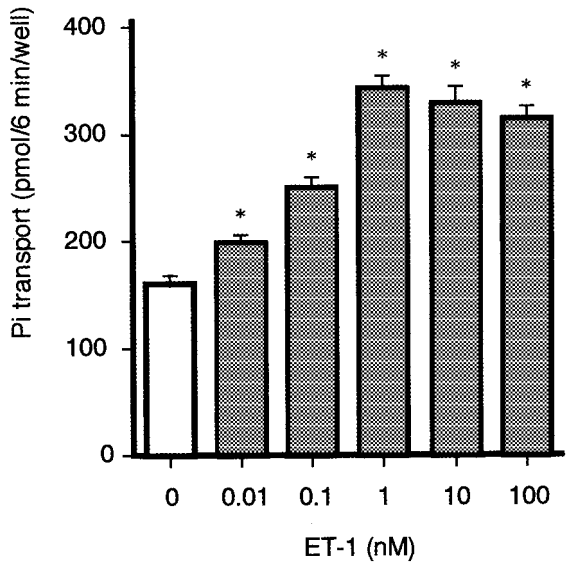

Fig. 1. Dose-dependent effect of ET-1 on Pi transport activity in MC3T3-E1 osteoblast-like cells. MC3T3-E1 cells cultured for 4 days were switched to α-MEM containing 2% FCS for 24 h. Then, they were incubated for 6 h in the same culture medium with either various doses of ET-1 or its vehicle. Pi uptake was determined in EBSS containing 0.1 mM $^{32}PO_4$. Each value represent mean±SEM of five to six determinations from a representative experiment. $^*P < 0.01$, compared with vehicle (ANOVA, Fischer test).

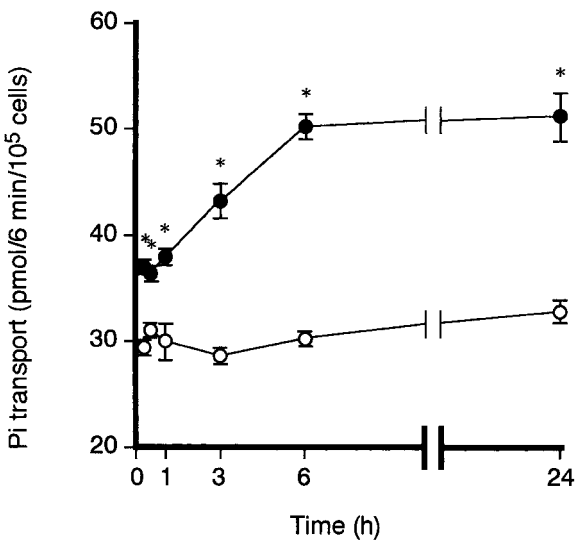

Fig. 2. Time-dependent effect of ET-1 on Pi transport in MC3T3-E1 osteoblast-like cells. MC3T3-E1 cells cultured for 4 days were switched to α-MEM containing 2% FCS for 24 h. Then, they were incubated with either 10 nM ET-1 (●) or its vehicle (○) for various periods. Pi uptake was determined in EBSS containing 0.1 mM $^{32}PO_4$. Each value represents mean±SEM of five to six determinations from a representative experiment. $^*P < 0.01$, compared with vehicle (Unpaired Student's *t*-test).

TABLE I. Effect of Transcriptional and Translational Inhibitors on Pi Transport Stimulation Induced by ET-1 in MC3T3-E1 Osteoblast-Like Cells

	Pi transport (pmol/6 min/well)
Vehicle	78.0±3.0
ET-1	152.7±2.8
Actinomycin D	51.3±3.0
Actinomycin D + ET-1	56.4±4.1
Cycloheximide	60.8±2.0
Cycloheximide + ET-1	62.1±1.5

Confluent cells were cultured for 24 h in α-MEM containing 2% FCS. They were then pretreated with either 5 μg/ml of actinomycin D or 2 μM of cycloheximide for 3 h, and then incubated with either 10 nM ET-1 or its vehicle for 6 h. Pi uptake was determined in EBSS containing 0.1 mM $^{32}PO_4$. Each data represents mean±SEM of five to six determinations from representative experiments.

1 (10 nM) was due to alteration of the maximal velocity (V_{max}) of the Pi transport system (vehicle, 0.37±0.03 nmolPi/6 min/well; ET-1, 0.64±0.01 nmol Pi/6 min/well; P value was < 0.01) with no significant change of the affinity constant (Km) of the carrier for Pi (vehicle, 0.45±0.07 mM; ET-1, 0.47±0.02 mM). Pretreatment of the cells with either 5 μg/ml actinomycin D or 2 μM cycloheximide for 3 h significantly reduced Pi transport stimulation induced by 10 nM ET-1 (Table I).

Signaling Mechanism Involved in ET-1 Stimulation of Pi Transport

To examine which receptor is involved in the stimulation of Pi transport induced by ET-1, we tested the effect of BQ123, a more selective ET_A receptor antagonist [Ihara et al., 1992], and BQ788, a more selective ET_B receptor antagonist [Ishikawa et al., 1994], on Na-dependent Pi transport in MC3T3-E1 cells. BQ123 (IC_{50} for ET_A receptors = 22 nM), which alone did not affect basal levels, signficantly inhibited the ET-1-induced Pi transport in a dose-dependent manner in the range between 22 nM (IC_{50}) and 22 μM ($IC_{50} \times 1000$) in MC3T3-E1 cells, while BQ788 (IC_{50} for ET_B receptors = 1.2 nM) had little effect on the ET-1-induced Pi transport in the range between 1.2 nM (IC_{50}) and 1.2 μM ($IC_{50} \times 1000$) in these cells (Fig. 3).

ET-1 has been reported to stimulate at least three phospholipases, PLA_2, PLC, and PLD through ET_A receptor in MC3T3-E1 cells [Suzuki et al., 1997]. To explore intracellular signaling system involved in ET-1-induced

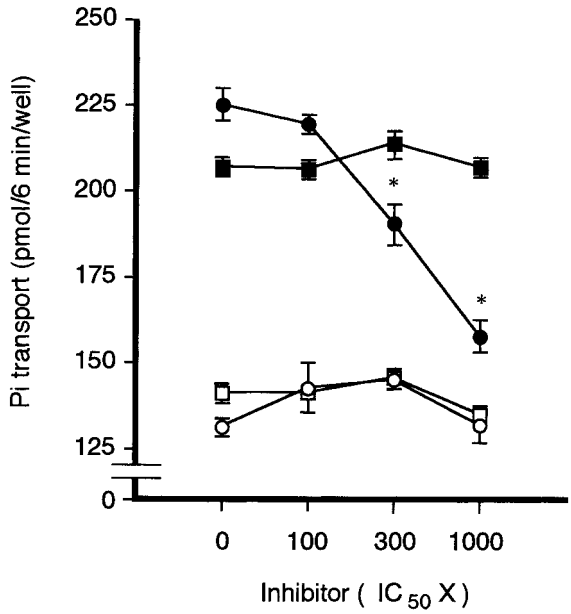

Fig. 3. Effects for BQ123 or BQ788 on the ET-1-induced formation of Pi transport in MC3T3-E1 cells. MC3T3-E1 cells cultured for 4 days were switched to α-MEM containing 2% FCS for 24 h. They were pretreated with various doses of BQ123 (IC_{50} for ET_A receptors = 22 nM) (○,●) or BQ788 (IC_{50} for ET_B receptors = 1.2 nM) (□,■) for 1 h, and then stimulated by 10 nM ET-1 (●,■) or vehicle (○,□) for 6 h. Each value represent the mean±SEM of five to six determinations from a representative experiment. *$P < 0.01$, compared with the value of ET-1 alone (ANOVA, Fischer test).

enhancement of Pi transport in osteoblast-like cells, we examined the effect of U73122, an inhibitor of PI-PLC, and U73343, an inactive form of U73122 for PI-PLC inhibition [Bleasdale et al., 1990]. U73122 (20 μM), which did not affect basal levels, partially but significantly inhibited the ET-1-induced Pi transport, while U73343 showed little effect on the ET-1-induced Pi transport (Table II).

PC hydrolysis by PLD results in the formation of choline and phosphatidic acid (PA), which further degraded by PA phosphohydrolase to diacylglycerol (DAG) [Exton, 2000]. To examine whether the signaling pathway via PC hydrolysis by PLD involved in the stimulation of Pi transport induced by ET-1 in osteroblast-like cells, we tested the effects of propranolol, an inhibitor of PA phosphohydrolase [Pappu and Hauser, 1983] on Pi transport in MC3T3-E1 cells. As shown in Figure 4A, Pi transport stimulation by ET-1 was dose-dependently inhibited by propranolol with a maximal effect at 200 μM. On the contrary, indomethacin, a

TABLE II. Effect of U73122 and U73343 on ET-1-Induced Pi Transport in MC3T3-E1 Osteoblast-Like Cells

	Pi transport (% Control ± SEM)
Vehicle	100.0±4.6
U73122	105.5±2.6
U73343	103.9±1.1
ET-1	187.8±2.8
U73122 + ET-1	136.4±2.0*
U73343 + ET-1	177.7±5.2

Confluent MC3T3-E1 cells were cultured for 24 h in α-MEM containing 2% FCS. They were pretreated with either 20 μM of U73122, U73343, or their vehicle for 1 h, and then incubated with 10 nM ET-1 or its vehicle for 6 h. Pi uptake was determined in EBSS containing 0.1 mM $^{32}PO_4$. Each data represent mean±SEM of five to six determinations from a representative experiment.
*$P < 0.01$, compared with ET-1-treated cells without inhibitor (ANOVA, Fischer test).

TABLE III. Combined Effect of PMA and ET-1 on Pi Transport in MC3T3-E1 Osteoblast-Like Cells

	Pi transport (pmol/6 min/well)
Vehicle	62.6±6.4
ET-1	121.0±8.8*
PMA	157.6±3.7*
PMA + ET-1	167.7±4.9*

Confluent cells were cultured for 24 h in α-MEM containing 2% FCS. They were then incubated with either 10 nM ET-1, 1 μM PMA or its vehicle for 6 h. Pi uptake was determined in EBSS containing 0.1 mM $^{32}PO_4$. Each data represents mean±SEM of five to six determinations from representative experiments.
*$P < 0.01$ compared with vehicle.

cyclooxygenase inhibitor, did not affect either basal or ET-1-induced Pi transport in MC3T3-E1 cells (Fig. 4B).

As both PI hydrolysis by PLC and PC hydrolysis by PLD result in the activation of PKC, we next examined the involvement of PKC in ET-1-induced Pi transport activation in MC3T3-E1 cells. PMA, a PKC-activating phobol ester [Nishizuka, 1995], stimulated Na-dependent Pi-transport in MC3T3-E1 cells, and the combination of PMA and ET-1 did not enhance Pi transport further (Table III). Calphostin C, a potent and selective PKC inhibitor, dose-dependently suppressed ET-1-induced Pi transport in the range between 0.03 and 1 μM (Fig. 5A). In addition, ET-1-induced Pi transport was markedly attenuated in PKC-down regulated cells compared to that in control cells (Fig. 5B).

It has been reported that ET-1 stimulates p42/44 and p38 MAP kinases in MC3T3-E1 cells [Kawamura et al., 1999a, b]. U0126, a specific inhibitor of MEK [Favata et al., 1998], an upstream kinase of p42/44 ERK MAP kinase, did not affect ET-1-induced Pi transport activity. In addition, 10 μM of p38MAPK inhibitor

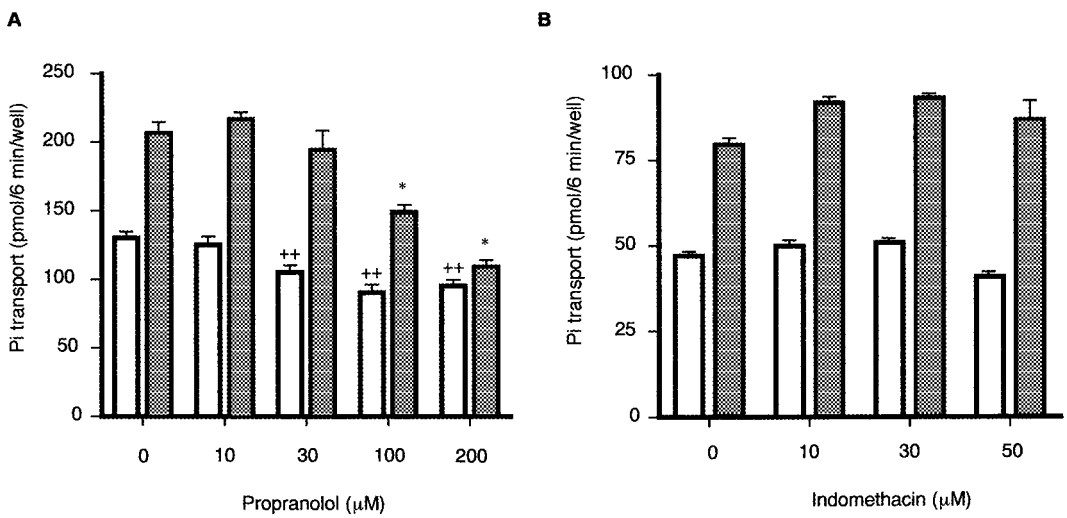

Fig. 4. Effect of propranolol (**A**) or indomethacin (**B**) on Pi transport stimulation induced by ET-1 in MC3T3-E1 osteoblast-like cells. MC3T3-E1 cells cultured for 4 days were switched to α-MEM containing 2% FCS for 24 h. They were then pretreated with various doses of propranolol (A), indomethacin (B) or their vehicle for 1 h, and then incubated with either 10 nM ET-1 (▨) or its vehicle (□) for 6 h. Pi uptake was determined in EBSS containing 0.1 mM $^{32}PO_4$. Each value represents mean±SEM of five to six determinations from a representative experiment. *$P < 0.01$ compared with the value of ET-1 alone (ANOVA, Fischer test) ++$P < 0.01$ compared with vehicle (ANOVA, Fischer test).

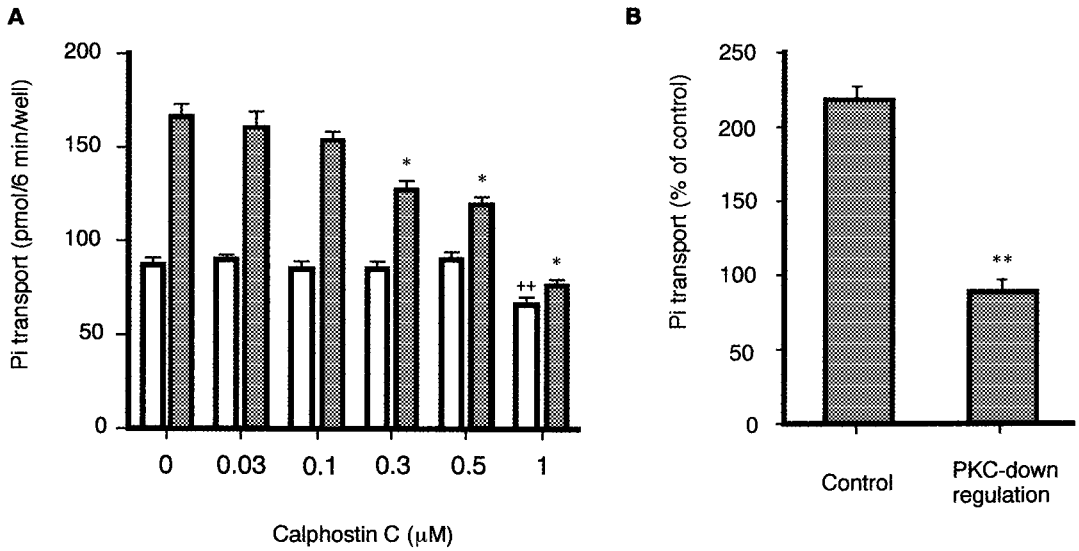

Fig. 5. Effect of calphostin C (**A**) or PKC down regulation (**B**) on Pi transport stimulation induced by ET-1 in MC3T3-E1 osteoblast-like cells. MC3T3-E1 cells cultured for 4 days were switched to α-MEM containing 2% FCS for 24 h. They were pretreated with various doses of calphostin C for 1 h (A) or with 10 μM of PMA or its vehicle for 24 h (B). Then, they were incubated with either 10 nM ET-1 (▨) or its vehicle (□) for 6 h. Pi uptake was determined in EBSS containing 0.1 mM $^{32}PO_4$. Each value represents mean±SEM of five to six determinations from a representive experiment. In panel (B), the data was expressed as percentage of the value of vehicle-treated cells in each experimental condition. $^{*}P < 0.01$ compared with the value of ET-1 alone (ANOVA, Fischer test) $^{**}P < 0.01$ compared with ET-1 in control cells (Unpaired Student's *t*-test) $++P < 0.01$ compared with vehicle (ANOVA, Fischer test).

SB203580 [Cuenda et al., 1995] had little effect on the ET-1-induced enhancement of Pi transport (data not shown).

DISCUSSION

ET has been reported to modulate the cellular functions of bone-forming cells [Zaidi et al., 1993; Stern et al., 1995], and both catabolic and anabolic effects of ET-1 on bone tissue have been reported [Tatrai et al., 1992; Shioide and Noda, 1993; Zaidi et al., 1993; Stern et al., 1995; Hiruma et al., 1998; Nelson et al., 1999]. In the present study, we investigated the effect of ET-1 on Pi transport, which is considered to play an important role in the mechanism of calcification in bone tissues [Caverzasio et al., 1996], and its intracellular signaling pathways in osteoblast-like MC3T3-E1 cells. We here showed that ET-1 induced time- and dose-dependent stimulation of Na-dependent Pi transport in these cells. On the other hand, ET-1 did not stimulate alanine transport, which is also driven by the transmembrane gradient of Na, suggesting the selective stimulatory effect of ET-1 on Na-dependent Pi transport in osteoblast-like cells. Our findings that both actinomycin D and cycloheximide inhibited the ET-1-induced stimulation of Pi transport suggest that the change in Pi transport activity induced by ET-1 depends on both transcriptional and translational process. The stimulatory effect of ET-1 also corresponds to a change in the V_{max} of the Pi transport system, while Km value was not affected by ET-1. These observations suggest that enhanced Pi transport activity in response to ET-1 may results from insertion of newly synthesized Pi transporters into the plasma membrane. The exact role of ETs in bone formation and resorption is still controversial. Although, Hiruma et al. [1998] has reported that ET inhibits the mineralization of osteoblastic cells in vitro, another group has recently reported that ET-1 stimulates both proliferation and the formation of bone nodules in osteoblastic progenitor cells [von Schroeder et al., 2000]. In addition, it has been reported that ET-1 overexpression induced new bone formation in osteoblastic tumor model [Nelson et al., 1999]. Our findings in this study suggest that it is probable that ET has positive effect on the initiation of calcification of bone matrix.

Next, we examined the intracellular signaling mechanism involved in ET-1-induced stimulation of Na-dependent Pi transport in MC3T3-E1 cells. There are at least three types

of ET receptors, namely ET_A, ET_B, and ET_C [Pollock et al., 1995]. In this study, we showed that not BQ788, a selective ET_B receptor antagonist, but BQ123, a selective inhibitor for ET_A receptor, blunted the stimulation of Pi transport induced by ET-1 in MC3T3-E1 cells. The result indicated that ET_A receptor was the selective receptor in the stimulation of ET-1-induced Pi transport in osteoblast-like cells. In MC3T3-E1 cells, it has been reported that ET_A receptor mediates at least three intracellular signaling pathways of ET-1: (1) PI hydrolysis by PLC; (2) PC hydrolysis by PLD; (3) arachidonic acid release by PLA_2 [Suzuki et al., 1997]. In the present study, we explored if these signaling pathways are involved in ET-1-induced enhancement of Pi transport in MC3T3-E1 cells. PI hydrolysis by PLC results in the production of two second messengers, inositol 1,4,5-trisphosphate, which mobilizes Ca^{2+} from intracellular Ca store, and DAG, a physiological PKC activator [Berridge, 1995]. In this study, we showed that PI-PLC inhibitor, U73122 (20 μM), partially but significantly reduced ET-1-induced Pi transport, while U73343, an inactive control of U73122 for PI-PLC inhibition at 20 μM, did not affect either basal or ET-1-induced Pi transport in MC3T3-E1 cells. These findings suggest that ET-1-induced PI hydrolysis by PI-PLC takes part in the stimulatory effect of ET-1 on Pi transport in osteoblast-like cells. PC is the principal phospholipid in the cell membranes, and it is hydrolyzed by PC-PLD, resulting in the formation of PA [Exton, 2000]. PA, which itself could be a potential intracellular mediator, can be further degraded by PA phosphohydrolase to DAG. It is now considered that DAG formation resulting from PC hydrolysis by PLD is important for sustained activation of PKC [Exton, 2000]. Our present result that an inhibition of degeneration from PA to DAG by propranolol significantly reduced ET-1-induced Pi transport in MC3T3-E1 cells suggested the involvement of DAG delivered from PLD hydrolysis in the signaling cascade of ET-1-induced Pi transport in osteoblast-like cells. Arachidonic acid released by PLA_2 is further catalyzed by cyclooxegenase to prostaglandins, leukotrienes, and thromboxanes, which act as an autocrine and paracrine factors in ubiquitous cells. Among them, prostaglandin E_2 has been reported to stimulate Na-dependent Pi transport in osteoblast-like cells [Veldman et al., 1998]. However, we here showed that indomethacin, an inhibitor for cyclooxygenase, affected neither basal nor ET-1-induced Pi transport in MC3T3-E1 cells. This results suggests that it is unlikely that prostaglandins which is resulted from arachidonic acid release mediate the stimulatory effect of ET-1 on Pi transport in osteoblast-like cells.

Both PI-PLC and PC-PLD hydrolyses result in the formation of DAG, which activates PKC [Nishizuka, 1995; Exton, 2000]. We next examined whether PKC mediates the stimulation of Pi transport activity by ET-1 in MC3T3-E1 cells. PMA, a PKC-activating phorbol ester, also stimulated Na-dependent Pi transport in MC3T3-E1 cells, and the combined effect of PMA and ET-1 on Pi transport was not additive. This finding suggests the existence of common pathway to stimulate Pi transport for PMA and ET-1 in these cells. In addition, we showed that PKC specific inhibitor, calphostin C, significantly blunted ET-1-induced Pi transport, suggesting the significant role of PKC to induce Pi transport by ET-1 in osteoblast-like cells. Furthermore, we showed that ET-1 did not enhance Pi transport in PKC down-regulated MC3T3-E1 cells. Our results as a whole suggest that PKC activation by ET-1 plays an important role in ET-1-induced enhancement of Pi transport activity in osteoblast-like cells.

The involvement of MAP kinases in several cellular functions of ET-1 in MC3T3-E1 cells has been reported [Kawamura et al., 1999a,b]. In this study, we found that U0126, a specific inhibitor of MEK which activates p42/44 ERK MAP kinase, had no effect on the enhancement of Pi transport by ET-1 in MC3T3-E1 cells. In addition, p38 MAP kinase inhibitor SB203580 did not affect ET-1-induced Pi transport in these cells at the concentration (10 μM) where SB203580 specifically inhibits p38 MAP kinase. These findings suggest that it seems unlikely that ET-1-induced enhancement of Pi-transport is mediated by these MAP kinases.

In conclusion, the results of the present study indicate that, in MC3T3-E1 osteoblast-like cells, ET-1 acting through ET_A receptor links to a stimulation of Pi transport via activation of PKC through both PI and PC hydrolyses.

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