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Calcium and Insulin-like Growth Factor I Stimulation of Sodium-Dependent Phosphate Transport and Proliferation of Cultured Rat Osteoblasts

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Calcium (Ca) stimulates proliferation of osteoblasts in vitro, an effect proposed to be mediated by IGF I. Addition of 1 mM Ca or of 1 nM IGF I to the medium (0.3 mM Ca) of a rat bone-derived cell line, PyMS, stimulated not only DNA synthesis but also sodium-dependent (Na_d) phosphate (Pi) uptake, the latter, within 2 h. These cells barely express and produce IGF I. IGF binding protein-3 which inhibits IGF action decreased neither basal nor Ca-stimulated but IGF I-stimulated Na_dPi transport and DNA synthesis, indicating that Ca stimulated Na_dPi transport and DNA synthesis independently of IGF I. The effects of Ca and IGF I on DNA synthesis were additive. 1 μM nifedipine blocked IGF I- and Ca-stimulated DNA synthesis but not Na_dPi transport, suggesting that Ca influx is not mediating the Na_dPi transport-enhancing IGF I signal but is required for IGF I-induced osteoblast proliferation. © 1998 Academic Press

Most of the calcium (Ca) in the body is stored in the skeleton in the form of apatite. At sites of bone resorption, osteoclasts dissolve mineralized bone matrix and release Ca into the extracellular fluid, where it may play a physiological role in bone remodeling. As a first messenger, Ca could act through cell surface receptors to stimulate the proliferation and recruitment of bone-forming osteoblasts. Recently, Ca^{2+}-sensing receptors have been identified in parathyroid glands, kidneys, and brain (1). Cation-sensing receptors that are functionally similar but molecularly distinct from Ca^{2+}-sensing receptors are expressed in murine-derived MC3T3-E1 (pre)osteoblasts (2,3). Ca is a mitogen for these mouse cells (3,4): It has been reported that high (5 mM) Ca stimulated not only DNA synthesis but also IGF I production. Stimulation by Ca of DNA synthesis could be blocked by IGF I antiserum or by antibodies directed against the type 1 IGF receptor, suggesting that the Ca effect was mediated by IGF I (4). Furthermore, raising the extracellular Ca concentration by 0.8-2 mM increased proliferation and the concentration of IGF II in the culture medium of human HBV155 osteoblastic cells (5). In that study, IGF II was considered important for the stimulatory effect of Ca, because monoclonal antibodies against IGF II abolished the Ca response.

We have previously found that IGF I stimulated Na_dPi transport and DNA synthesis in PyMS cells, a (pre)osteoblastic rat cell line which is very sensitive to stimulatory effects of low IGF I concentrations (6,7). IGF binding protein (BP)-3 which sequesters and specifically blocks locally produced IGFs in osteoblast cultures decreased DNA synthesis in rat calvarial but not in PyMS cells suggesting that the role of autocrine/paracrine IGF in PyMS cells is unessential (7).

In the present study, we examined the effects of increasing extracellular Ca concentrations on Na_dPi transport, proliferation, and alkaline phosphatase activity in PyMS cells to test whether the effects of Ca are similar to those of IGF I, and whether locally produced IGFs mediate the effects of Ca. Furthermore, using nifedipine, a pharmacological blocker of Ca channels, we tested whether Ca influx into the cells is required for Ca and IGF I to stimulate Na_dPi transport and DNA synthesis in PyMS cells.

MATERIALS AND METHODS

Cell cultures, test compounds and incubations. PyMS cell cultures between passages 11 and 27 were used. Some experiments were also performed with newborn rat calvarial bone-derived cells, (6,7). Cells were plated in multiwell tissue culture plates (Falcon, 35 mm diameter) at a density of 2×10^5 cells/well. Three days later, confluent monolayers had formed, and cells were exposed to serum-

Abbreviations: Ca, calcium; IGF I, insulin-like growth factor I; IGFBP-3, insulin-like growth factor binding protein-3; Na_d, sodium-dependent; Pi, phosphate.

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free Ham's F-12 medium containing gentamicin (50 μg/ml), glutamine (2 mM) and charcoal-treated bovine serum albumin (BSA, from Serva) at 1 g/l. RhIGF I (Ciba-Geigy, Basel, Switzerland), $CaCl_2$, and $MgCl_2$ (from Sigma) were added to the test medium as specified in the result section. For the experiments presented in Table 2, cell monolayers were incubated for 10 min with nifedipine (Calbiochem, La Jolla, CA) and rhIGFBP-3 (kind gift from Dr. A. Sommer, Celtrix, Santa Clara, CA), and aliquots of $CaCl_2$ and rhIGF I were added 10 min later. Nifedipine was dissolved in absolute ethanol at a concentration of 10 mM (final concentration of 1 μM) and all media were adjusted to a final alcohol concentration of 0.01% (vol/vol).

Phosphate and alanine transport studies, determination of alkaline phosphatase activity, protein content, cell number, ^{3}H-thymidine incorporation into DNA, and statistical analysis. Transport studies were carried out at room temperature in buffer containing 140 mM NaCl or in buffer containing choline chloride instead of NaCl for measuring Na^+-independent solute uptake (6). Alkaline phosphatase activity and protein content were determined in 0.1% Triton X-100 lysates. Cell number from parallel dishes was counted after detachment with trypsin (6). Parallel dishes were exposed to test medium for 18 h and pulsed with methyl-^{3}H-thymidine (Amersham, 80 Ci/mmol; 1 μCi/dish) for 3 h at 37°C (6,7).

Results are represented as means ± SE and analyzed for differences by Student's *t* test.

Radioimmunoassay for IGF I, Northern hybridization for type 1 IGF receptor and IGF I mRNA, and Western blot analysis for IGFBPs. In some of the experiments, test media were collected after 24 h and IGF I was extracted by Sep-Pak C18 cartridges and determined using rabbit antiserum (1:20′000) against human IGF I and pure rat IGF I (gift from Dr. M. Kobayashi, Fujisawa, Japan) as a standard (8). RNA was prepared from cells grown on 10 cm diameter plates and from calvarial bones, as described elsewhere (8,9). For isolation of RNA from bone, parietal bones were carefully dissected from 100 newborn rats. Total RNA and poly(A)$^+$ RNA were size-fractionated in formaldehyde-containing agarose gels, and the blots hybridized to a cDNA encoding part of the β-subunit of the mouse type 1 IGF receptor (kind gift of Dr. M. Böni-Schnetzler) and a rat IGF I cDNA (8,9). To check whether rhIGFBP-3 was degraded during the incubations, media were analyzed for the recovery of added rhIGFBP-3 after 24 h by ^{125}I-IGF II ligand blot or immunoblot using polyclonal IGFBP-3 antiserum raised in rabbits (gift of Dr. A. Sommer, Celtrix, Santa Clara, CA) (7).

RESULTS

Na_dPi transport increased linearly with incubation time in control- (Figure 1A) and Ca-treated cells (Figure 1B) for up to 20 minutes and was elevated in PyMS cells exposed to increasing concentrations of Ca for 24 h (Figure 2A). Ca stimulated Na_dPi transport to the same extent whether Pi concentration was 0.1 mM (Figure 2A), i.e. a concentration close to the K_M of the Na_dPi transport system, or 1 mM (not shown), i.e. a concentration closer to the physiological extracellular Pi concentration. Increasing the Ca concentration by 0.1 mM to a final concentration of 0.4 mM produced a significant stimulation of Na_dPi transport (Figure 2A). Maximal stimulation was observed after 24 h at a final concentration of 1.3 mM (Figure 2A). Similar results were obtained using normal rat calvaria cells (not shown). Addition of 1 mM Mg had no effect on Na_dPi transport. The kinetics of the Ca effect on Na_dPi transport were obtained by measuring transport over a range of Pi concentrations from 0.02 to 2 mM. Lineweaver-Burk plot analysis showed that Ca increased the v_{max} of the Na_dPi transport system from 1.35±0.05 to 1.96±0.08 nmol Pi/mg protein × 10 min, whereas the K_M was not affected (108±3 μmol/l after one day in low Ca and 112±6 μmol/l after one day in high Ca). 1.3 mM Ca increased Na_dPi transport within 2 h from 0.98±0.06 (control) to 1.18±0.03 (1.2-fold, $p<0.05$), within 6 h to 1.29±0.03 (vs. 1.01±0.06 in control, 1.3-fold) and to 1.59±0.03 nmol Pi/mg protein × 10 min (vs. 1.07±0.07, 1.5-fold) after 24 h in 3 independent time course experiments (in triplicate). Ca did not stimulate Na_d alanine uptake after 2, 6 (not shown) and 24 h (Table 1). Addition of Ca stimulated DNA synthesis in a dose-dependent manner and exerted similar effects in normal rat calvaria cells (not shown). The lowest added Ca concentration that produced significant stimulation was 0.1 mM, and maximal stimulation occurred after the addition of 1 mM (Figure 2B). 1 mM Ca did not affect cell number, protein content or alkaline phosphatase activity after 24 h of treatment (Table 1).

IGF I at 1 nM stimulated Na_dPi transport 2.7-fold and DNA synthesis 8.5-fold (Table 2). Neither 1 nM IGF I alone nor in combination with 1 mM Ca significantly altered protein content, alkaline phosphatase activity and cell number (Table 1). Their effects on DNA synthesis but not those on Na_dPi transport were additive (Table 2). When tested in the absence of rhIGF I, IGFBP-3 did not affect Na_dPi transport or ^{3}H-thymidine incorporation into DNA (7), as also shown in Table 2A. The stimulatory effects of 1 nM IGF I on Na_dPi transport and DNA synthesis but not those of 1 mM Ca were blocked by 10 nM (Table 2A) or 100 nM (not shown) IGFBP-3. Although some decrease in the rhIGFBP-3 concentration can be observed in the medium (7), intact IGFBP-3 remains in >100-fold molar excess over the IGF I concentrations (see below). Ca did not increase the disappearance rate of IGFBP-3 from the medium.

Northern blot analysis showed the strongest hybridization signal with the type 1 IGF receptor cDNA (mainly an 11 kb transcript) in the PyMS cells (Fig. 3, left). Correspondingly, PyMS cells exhibit ~5 times more specific IGF I binding sites than rat calvarial cells, as estimated by ^{125}I-IGF I competition binding and Scatchard transformation studies, whereas their affinity is similar, ~0.5 nM (C.S., unpublished). In contrast, IGF I mRNA (mainly a 7.5 kb transcript) was most abundant in cultured calvarial cells. A weak hybridization signal (even less than with RNA from bone) could be detected in PyMS cells only in the poly(A)$^+$ RNA lane upon prolonged exposure of the filter to the X-ray film. Consistent with the low level of IGF I mRNA expression, IGF I remained below the limit of immunoassay detection in the medium of PyMS cells throughout a 24 h test period, also in the presence of 1 mM additional Ca.

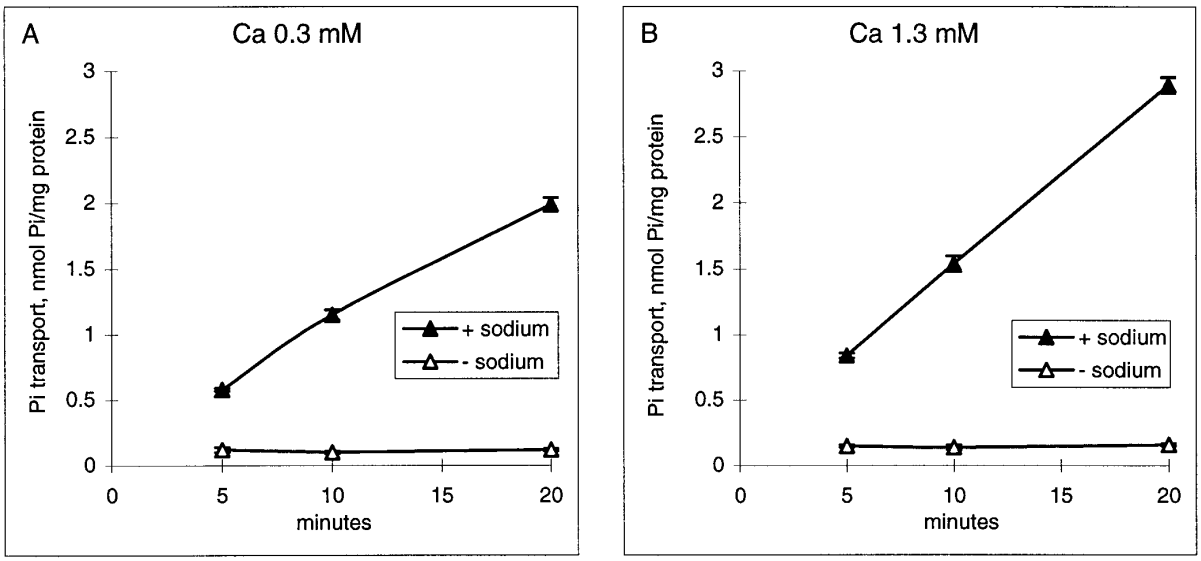

FIG. 1. Time course of Pi uptake by PyMS cells exposed for 24 h to control (0.3 mM Ca, A) and Ca-supplemented (1.3 mM, B) medium in Na- or in choline-containing buffer. Cells were exposed to serum-free medium containing 0.3 mM (control, A) or 1.3 mM (B) Ca, then incubated in the presence of sodium chloride (circles) or choline chloride (triangles) buffer. Na_dPi transport was measured per 5, 10 or 20 min. Pi concentration was 0.1 mM. Data shown are means ± SE of a representative experiment in triplicate.

Because Ca may not only be a first (extracellular) but also a second (intracellular) messenger, we examined the effects of nifedipine, a pharmacological blocker of Ca channels. Nifedipine at 1 μM did not significantly affect basal Na_dPi transport and ^{3}H-thymidine incorporation into DNA and did neither prevent IGF I nor Ca-stimulated Na_dPi transport, but it blocked IGF I- and Ca-stimulated DNA synthesis (Table 2B).

DISCUSSION

Ca stimulated Na_dPi transport in PyMS (and normal calvaria) cells but did not affect Na_d-alanine transport,

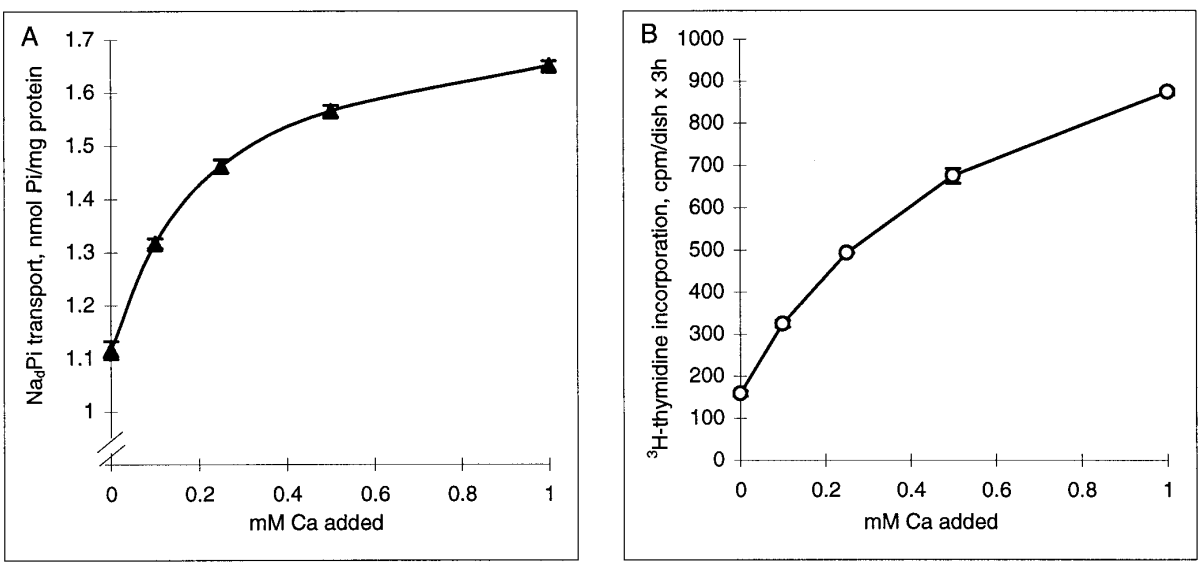

FIG. 2. Dose-response curve of Na_dPi transport (A) and ^{3}H-thymidine incorporation into DNA (B) in PyMS cells exposed to increasing Ca concentrations. A: Cells were incubated in serum-free medium (containing 0.3 mM Ca) to which Ca was added as indicated for 24 h before measuring Na_dPi transport for 10 min. Pi concentration was 0.1 mM. B: Cells were exposed to increasing Ca concentrations for 18 h, pulsed with ^{3}H-methyl-thymidine for 3 h, and incorporation of ^{3}H-thymidine into DNA was measured. For both (A) and (B), the data represent means of ± SE of 3 experiments in triplicate. Significance (*, $p<0.05$) is indicated in comparison with vehicle values.

TABLE 1

Cell Number, Protein Content, Alkaline Phosphatase Activity and Na_d Alanine Transport in PyMS Cells Exposed to Additional Ca in the Presence/Absence of 1 nM rhIGF I for 24 h

	Protein content, μg/dish	ALP activity, μmol/dish × h	Cell number, 10^5/dish	Na_d alanine transport, pmol alanine/mg protein × 10 min
Control	113 ± 4	0.467 ± 0.015	3.02 ± 0.14	328 ± 6
0.25 mM Ca	118 ± 3	0.461 ± 0.012	3.03 ± 0.14	331 ± 8
1 mM Ca	114 ± 6	0.466 ± 0.011	3.20 ± 0.07	333 ± 6
1 nM IGF I	130 ± 3	0.538 ± 0.023	3.29 ± 0.16	352 ± 8
0.25 mM Ca + 1 nM IGF I	128 ± 5	0.542 ± 0.020	3.39 ± 0.12	350 ± 4
1 mM Ca + 1 nM IGF I	130 ± 8	0.535 ± 0.022	3.39 ± 0.11	355 ± 6

Note. Values represent the mean ± SE, from 3 (protein content, ALP activity, Na_d alanine transport) or 4 (cell number) separate experiments in triplicate. No significant differences for all comparisons.

another Na_d transport system. Ca also stimulated DNA synthesis of PyMS (and normal calvaria) cells, confirming reports describing Ca (but not Mg) stimulation of DNA synthesis in mouse MC3T3-E1 (3,4) and human HBV155 (5) osteoblastic cells. Ca stimulates proliferation not only of bone but also of fibroblastic cells, such as mouse Balb/c/3T3 cells (10) and human fibroblasts (11). Studies using the pharmacological (but also toxic) agent Gd as a Ca-sensing receptor agonist (not shown) did not allow us to clarify to what extent the Ca effects reflect Ca sensing or Ca dependency of the cells.

In order to reassess previously reported findings in mouse (4) and human (5) osteoblast-like cells, we checked whether IGF mediates the Ca effect on Na_dPi transport and DNA synthesis in the PyMS bone cell line. In contrast to normal rat calvarial cells (8), PyMS cells barely produce IGF I. Concentrations in media were below 0.01 nM after 24 h. The high sensitivity of PyMS cells towards IGF I in terms of stimulation of DNA synthesis (7) and Na_dPi transport (6) is consistent with the insignificant expression and production of IGF I and a particularly prominent expression of type 1 IGF receptors, ~150′000 per cell. Only in normal rat calvarial but not in PyMS cells does autocrine IGF I contribute to basal Na_dPi transport and basal DNA synthesis (7) (Table 2). According to our data in the two cell types, there is an inverse relationship between type 1 IGF receptor expression and sensitivity to added IGF I on one hand, and IGF I expression and sensitivity to the inhibitory action of added IGFBP-3 on the other hand. IGF I expression by normal rat calvarial cells grown in culture is higher than IGF I expression by osteoblasts in vivo (Figure 3), possibly reflecting adaptation to the culture conditions. In vitro studies may, therefore, overestimate IGF I production by bone cells.

Ca stimulated Na_dPi transport significantly within 2 h, and Ca and IGF I together were more effective in stimulating DNA synthesis than IGF I alone at supramaximal concentrations (Table 2), consistent with another report (3). Most importantly, IGFBP-3

TABLE 2

Na_dPi Transport and DNA Synthesis in PyMS Cells Exposed to Additional Ca and rhIGF I in the Presence of 10 nM rhIGFBP-3 (A) or 1 μM Nifedipine (B)

	Na_dPi transport, nmol Pi/mg protein × 10 min		^{3}H-thymidine incorporation, cpm ^{3}H/dish × 3h	
A:	control	IGFBP-3	control	IGFBP-3
control	1.51 ± 0.04	1.41 ± 0.03	174 ± 8	181 ± 5
1 mM Ca	2.98 ± 0.03*	3.04 ± 0.05*	1085 ± 82*	1065 ± 22*
1 nM IGF I	4.38 ± 0.18*	1.48 ± 0.03	1577 ± 100*	183 ± 6
B:	control	nifedipine	control	nifedipine
control	1.23 ± 0.05	1.05 ± 0.02	292 ± 8	258 ± 6
1 mM Ca	2.00 ± 0.03*	1.79 ± 0.05*	1234 ± 134*	250 ± 7
1 nM IGF I	2.91 ± 0.03*	2.67 ± 0.06*	2345 ± 48*	315 ± 27
1 mM Ca + 1 nM IGF I	3.03 ± 0.05*	2.76 ± 0.08*	3909 ± 273*	491 ± 99

Note. Cells were preincubated with 10 nM IGFBP-3 or 1 μM nifedipine for 10 min, and 1 mM Ca and/or 1 nM IGF I were then added. After incubation for further 24 h, Na_dPi transport was determined at a Pi concentration of 0.1 mM for 10 min, or, after 18 h of incubation, pulsed with ^{3}H-thymidine for 3 h, and incorporation of ^{3}H-thymidine into DNA was measured. Data presented are means ± SE of 4 experiments in triplicate.

* $p < 0.05$ vs control.

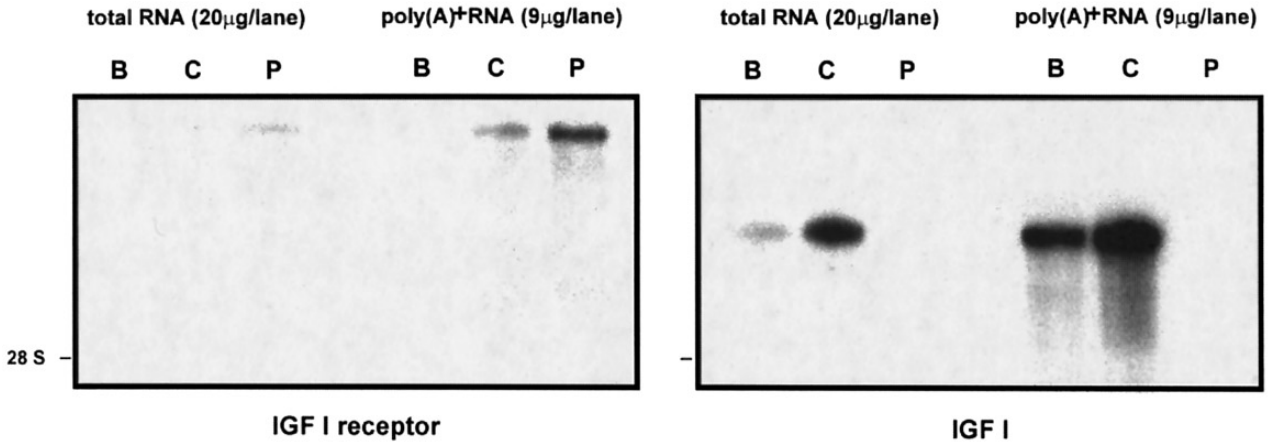

FIG. 3. Type 1 IGF receptor and IGF I mRNA in rat calvarial bone (B), rat calvarial bone-derived cultured cells (C), and in the rat calvarial bone-derived cell line, PyMS (P). The autoradiographs of the Northern blot analysis of total and poly(A)$^+$ RNA show hybridization signals with type 1 IGF receptor cDNA mainly at 11 kb and with IGF I cDNA mainly at 7.5 kb. 28 S, ribosomal RNA.

which blocks the stimulatory effects of IGF I and IGF II (7), did not affect the stimulation by Ca of Na_dPi transport and DNA synthesis (Table 2A). Therefore, autocrine IGF I is unlikely to mediate Ca-stimulated Na_dPi transport and DNA synthesis in PyMS cells, in contrast to findings in MC3T3-E1 cells (4).

We used nifedipine to see whether plasma membrane Ca channels are relevant in mediating increased Na_dPi transport and DNA synthesis in response to Ca and IGF I. The L-type Ca channels which are blocked by nifedipine have recently been found to be involved also in mechanotransduction in osteoblasts (12). 1 μM nifedipine blocked Ca- and IGF I-stimulated DNA synthesis but not Na_dPi transport (Table 2B), indicating that Ca channel-dependent pathways are required for the stimulation of DNA synthesis but not of Na_dPi transport. Again, these findings are at variance with those in mouse MC3T3-E1 cells where 10 μM nifedipine did not block the Ca-induced increase in DNA synthesis (4). However, IGF I was reported to activate a Ca-permeable cation channel in plasma membranes and to stimulate progression through the cell cycle in IGF I-responsive primed competent Balb/c/3T3 cells (13). Furthermore, IGF I-activated stimulation of 1α-hydroxylase and 1,25$(OH)_2D_3$ production in cultured mouse proximal tubular kidney cells was found to be blocked by 100 μM verapamil, another Ca channel blocker (14). Nevertheless, these data do not mean that Ca is the second messenger mediating all effects of IGF I. In our study, IGF I stimulated Na_dPi transport more rapidly than Ca, and this effect was not blocked by nifedipine. Regarding DNA synthesis, Ca may mediate the stimulatory effect of IGF I, but the data could also mean that cross-talk between an IGF I-signaling pathway and an extracellular Ca-triggered pathway is required for DNA synthesis.

Possibly, the effect of Ca and the relevance of Ca influx in IGF I signaling depends on the cell type and the study conditions. In mouse Balb/c/3T3 cells, Ca may play a role not only as a competence factor (10) but also in the progression through the cycle (13). IGF I, a progression factor, supports differentiation whereas platelet-derived growth factor, a competence factor, inhibits differentiation of osteoblast-like cells (15). However, we did not observe significant changes of alkaline phosphatase activity in response to Ca and IGF I within 24 h (Table 1). This finding, along with the lack of an effect on alanine transport and protein content of the cell cultures, supports the specificity of the Ca effect on Na_dPi transport although it does not clarify whether IGF I and Ca exert similar or distinct long-term effects on differentiated functions of osteoblasts.

In conclusion, our data show for the first time that Ca specifically stimulates Na_dPi transport in osteoblastic cells. Auto-/paracrine IGF I is unlikely to play a role in the stimulatory effects of Ca on Na_dPi transport and DNA synthesis in PyMS cells. Because increased Ca entry is required for stimulation of DNA synthesis but not of Na_dPi transport, distinct mechanisms seem to be involved in stimulatory effects of Ca and IGF I on Na_dPi transport and DNA synthesis.

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