Membrane peptidases on human osteoblast-like cells in culture: hydrolysis of calcitonin and hormonal regulation of endopeptidase-24.11

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Five membrane peptidase activities have been identified on cultured human osteoblast-like cells. These consisted of the four exopeptidases aminopeptidase-A, aminopeptidase-N, aminopeptidase-W and carboxypeptidase-M, and the endopeptidase, endopeptidase-24.11. The presence of endopeptidase-24.11 was confirmed immunochemically by immunofluorescent staining and by enzyme-linked immunosorbent assay. The inclusion of phosphoramidon partially inhibited the hydrolysis of human calcitonin by a membrane fraction prepared from osteoblast-like cell membranes, thus implicating endopeptidase-24.11 in its inactivation. Another metallopeptidase also contributed sub-

stantially to calcitonin hydrolysis. Purified porcine endopeptidase-24.11 (1 μ g) was shown to hydrolyse calcitonin with a half-life of 23 min, which compared to a half-life of 0.5 min for substance P under similar conditions. Sequence data revealed that the initial site of hydrolysis of calcitonin was between residues Lys¹⁸ and Phe¹⁹. The expression of endopeptidase-24.11 by cultured osteoblast-like cells was shown to be modified by various agents: expression was decreased by phorbol 12-myristate-13-acetate (160 nM for 48 h) and increased in the presence of calcitonin (1.5 nM for 48 h) and 1,25-dihydroxyvitamin D₃ (0.01–1 μ M for 72 h).

INTRODUCTION

Membrane peptidases are widely located throughout the mammalian body (Kenny, 1986) and are believed to possess a diversity of functions depending on their location. For example, endopeptidase-24.11 (E-24.11; EC 3.4.24.11) is a well-characterized cell-surface peptidase with high efficiency in hydrolysing oligopeptides (for review see Kenny et al., 1987) and is identical to the leukocyte antigen CD10 or common acute lymphoblastic leukaemia antigen (CALLA) (for review see LeBien and McCormack, 1989). Its location in the central nervous system makes it ideally situated to terminate the synaptic action of neuropeptides (Barnes et al., 1992). At other locations, for example the kidney and the choroid plexus, E-24.11 may play a protective role (Kenny et al., 1987; Bourne et al., 1989). On neutrophils, E-24.11 has been shown to hydrolyse *N*-formyl-Met-Leu-Phe (Connelly et al., 1985), a chemotactic peptide initiating neutrophil migration into sites of infection and inflammation (Schiffmann et al., 1975).

There are many polypeptide growth factors present in and around the bone matrix (Canalis et al., 1988). Some are produced by the bone itself, for example interleukin-6 (Feyen et al., 1989), while others are of systemic origin, for example calcitonin and parathyroid hormone. Some of these peptides are known to be involved in the regulation of the different cell types within osseous tissue (Raisz, 1988). For example, calcitonin, a 32-amino-acid peptide, acts on bone osteoclasts repressing their resorptive activity, whereas parathyroid hormone (PTH) has the converse effect and stimulates bone resorption. In addition, bone tissue has been shown to be innervated with vasoactive intestinal peptide (VIP)-immunoreactive nerve fibres (Hohmann et al., 1986), and purified porcine E-24.11 has been shown to hydrolyse VIP *in vitro* (Barnes et al., 1991). E-24.11 has been identified on human marrow stromal cell lines, with the highest levels of

expression being found on those cells with an osteoblastic phenotype (Indig et al., 1990).

Here we show that human osteoblast-like cells in culture express at least five membrane peptidases and that one of these, E-24.11, hydrolyses calcitonin and responds to hormonal stimulation. Thus membrane peptidases may contribute to bone formation and resorption, and hence may play a role in Ca²⁺ homeostasis.

EXPERIMENTAL

Materials

Antibodies and lectins

The polyclonal antibody RP161 was generated in a rabbit immunized with pig E-24.11 and was shown to be monospecific (Barnes et al., 1991). Anti-rabbit fluorescein isothiocyanate (FITC)-conjugated IgG was obtained from the Sigma Chemical Company, Poole, Dorset, U.K. Both the rhodamine-conjugated concanavalin A and rhodamine-conjugated wheat germ agglutinin were gifts from Dr. J. Walker, University of Leeds, Leeds, U.K.

Substrates

Succinyl-Ala-Ala-Leu-nitroanilide and *Streptomyces griseus* aminopeptidase were kindly provided by Dr. S. Blumberg, University of Tel Aviv, Tel Aviv, Israel. Human calcitonin and substance P were obtained from Cambridge Research Biochemicals Ltd., Northwich, Cheshire, U.K. Human parathyroid hormone (PTH) was obtained from the Sigma Chemical Company. All other substrates were from sources as described previously (Howell et al., 1992).

Abbreviations used: ACE, peptidyl dipeptidase-A ('angiotensin-converting enzyme'); AP-A, aminopeptidase-A; AP-N, aminopeptidase-N; AP-W, aminopeptidase-W; CP-M, carboxypeptidase-M; Dip-F, di-isopropylfluorophosphate; DPP IV, dipeptidyl peptidase IV; E-24.11, endopeptidase-24.11; EMEM, Eagle's minimum essential medium; FITC, fluorescein isothiocyanate; MDP, membrane dipeptidase; PMA, phorbol 12-myristate-13-acetate; PTH, parathyroid hormone; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; VIP, vasoactive intestinal peptide.

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Inhibitors

Phosphoramidon was obtained from Peninsula Laboratories Europe Ltd., St. Helens, U.K. Enalaprilat (MK422; *N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-proline) and cilastatin (MK0791; monosodium *Z*-*S*-(6-carboxy-6-[[2,2-dimethyl-(*S*)-cyclopropyl]-carbonylamino]-5-hexenyl)-L-cysteine) were gifts from Dr. A. A. Patchett and Dr. H. Kropp respectively, both of Merck Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A. Amastatin and di-isopropylfluorophosphate (Dip-F) were purchased from the Sigma Chemical Company.

Culture of human osteoblast-like cells

Human osteoblast-like cells were cultured as described by Beresford and co-workers (Beresford et al., 1984) from explants of trabecular bone obtained from knee joints removed during replacement surgery. Explants were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) foetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). Under these conditions, the resulting cells have been shown to exhibit stable osteoblast-like phenotypic characteristics, including the production of type I collagen and osteocalcin, responsiveness to PTH and high constitutive alkaline phosphatase activity, which could be further increased by 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (Beresford et al., 1984; Gallagher et al., 1986). In our experiments confluent primary cultures were passaged into 3.5 cm diameter wells (5 × 10⁴ cells/well) or on to 2.5 cm² plastic slides (3.3 × 10⁴ cells/slide) and the cells maintained for 5–7 days in medium until they were pre-confluent. For experiments with 1,25(OH)₂D₃ (Hoffmann-La Roche), cell monolayers were rinsed three times with serum-free EMEM and then incubated with EMEM supplemented with 10% (v/v) charcoal-stripped foetal calf serum [prepared by the addition of Norit A charcoal (Aldrich Chemical Company Ltd., Gillingham, Dorset, U.K.) to foetal calf serum (4 g/100 ml); this was stirred for 1 h at 4 °C, centrifuged (8000 g, 40 min, 4 °C) and then the supernatant filter-sterilized] and various concentrations of 1,25(OH)₂D₃ (10⁻¹⁰–10⁻⁶ M).

Preparation of cell membranes

The cell culture medium was removed and the cell monolayers rinsed twice with 10 ml of phosphate-buffered saline (140 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4). The cells were then scraped off the substratum into 150 mM NaCl, 18 mM CaCl₂, 50 mM Tris/HCl (pH 7.4) (1 ml) and pressure-homogenized using a Parr Cell Disruption Bomb (Scientific and Medical Products Ltd., Manchester, U.K.). The bomb (pre-chilled on ice) was pressurized to 5520 kPa (800 lbf/in²), maintained at 0 °C for 10 min and the cells then discharged. The homogenate (1 ml) was then centrifuged (10 min, 1000 g) and the supernatant (0.95 ml) centrifuged again (90 min, 100000 g, 4 °C) to yield a crude membrane pellet. Membrane proteins were solubilized by resuspending the pellet in 100 µl of 150 mM NaCl, 1% (v/v) Triton X-100, 50 mM Tris/HCl (pH 7.4), mixed for 1 h at 4 °C and any insoluble material was removed by centrifugation (10 min, 8000 g, 4 °C). Samples (referred to as P2) were stored at -20 °C until required for assay.

Hydrolysis of calcitonin, parathyroid hormone and substance P

Solubilized membrane protein (2 µg) or purified porcine E-24.11 (1 µg) was incubated at 37 °C with calcitonin (0.05 mM), PTH (50 µM) or substance P (0.5 mM). Termination of hydrolysis was performed by boiling samples for 4 min. Following centrifugation (10 min, 8000 g) the supernatant (60 µl) was analysed by reverse-

phase h.p.l.c. on a µBondapak C₁₈ column and peptides detected with a fixed-wavelength detector (214 nm). Products of calcitonin and substance P hydrolysis were resolved with a 15 min linear gradient of acetonitrile (4.8–60%) in 0.08% H₃PO₄ at pH 2.5, followed by elution for 5 min with 60% acetonitrile in 0.08% H₃PO₄, pH 2.5. The column was eluted at 1.5 ml/min. Under these conditions, calcitonin exhibited a retention time of 12.6 min and that of substance P was 9.0 min. Products of PTH hydrolysis were resolved as for those of calcitonin, except the gradient of acetonitrile was from 4.8–45% instead of 4.8–60%. Under these conditions PTH exhibited a retention time of 4.0 min.

Enzyme assays

E-24.11 activity was assayed by a two-stage assay adopted for use on a microtitre plate by using succinyl-Ala-Ala-Leu nitro-anilide as substrate supplemented with *Streptomyces griseus* aminopeptidase, as described by Indig et al. (1989). ACE (peptidyl dipeptidase-A), membrane dipeptidase (EC 3.4.13.11; renal dipeptidase; MDP), aminopeptidase-P (EC 3.4.11.9; AP-P) and aminopeptidase-W (AP-W) were assayed by the h.p.l.c. methods of Turner et al. (1989), Hooper et al. (1987), Hooper and Turner (1988) and Gee and Kenny (1987) respectively. Carboxypeptidase-M (CP-M) was assayed using Hip-Lys (1 mM) as substrate and hippuric acid release quantified using h.p.l.c. with elution conditions as described for the ACE assay. Aminopeptidase-N (EC 3.4.11.2; AP-N), aminopeptidase-A (EC 3.4.11.7; AP-A) and dipeptidyl peptidase IV (EC 3.4.14.5; DPP IV) were assayed by fluorimetric methods as described previously (Fulcher and Kenny, 1983). All enzyme units are expressed as nmol of substrate hydrolysed per min.

Other methods

E.l.i.s.a. of E-24.11 and immunofluorescent staining were performed as described previously (Howell et al., 1991 and 1992 respectively). The sequencing of peptide products was performed using an Applied Biosystems 477A protein sequencer. The preparation of kidney microvillar membranes (referred to as P4) was as reported by Booth and Kenny (1974). Protein was assayed with a bicinchoninic acid protein assay kit (Sigma Chemical Company) using the manufacturer's recommended protocol.

Table 1 Membrane peptidase activities identified on osteoblast-like cell membranes and a comparison with those of pig kidney microvilli

See the Experimental section for details. Kidney microvillar activities marked with an asterisk (*) were taken from Bourne et al., 1989 and those marked with † were taken from Howell et al., 1992. N.D., not detectable.

Enzyme	Specific activity (nmol/min per mg of protein)		
	Osteoblast membranes (P2)	Pig kidney microvillar membranes (P4)	Ratio (%)
AP-A	60.00	126*	47.6
AP-N	24.00	524*	4.6
AP-P	N.D.	68*	—
AP-W	0.26	120*	0.2
CP-M	7.26	5.3†	137
DPP IV	N.D.	606*	—
E-24.11	13.65	283†	4.8
MDP	N.D.	121*	—
ACE	N.D.	11.7†	—

Figure 1 Immunofluorescent staining for E-24.11 on human osteoblast-like cells

Cells, grown on plastic slides, were fixed and then detected by immunofluorescent staining before (c and d) and after (a and b) permeabilization with 0.1 % Triton X-100. (a) and (c) used a primary polyclonal antibody (RP161) raised against purified E-24.11 which was detected by an FITC-conjugated goat anti-rabbit IgG. (b) and (d) used a mixture of concanavalin A- and wheat germ agglutinin-rhodamine conjugate. n, cell nucleus; white arrow, endoplasmic reticulum staining; black arrow, cell surface staining. Bar, 5 μ m.

RESULTS

Characterizing membrane peptidase activities on osteoblast-like cells

Five membrane peptidase activities were identified on osteoblast-like cell membranes (Table 1). Of these five membrane peptidase activities, four were exopeptidases (AP-A, AP-N, AP-W and CP-M) and the other was an endopeptidase, E-24.11. ACE was not detectable. The activities of these membrane peptidases were compared with those in renal microvillar membranes, which is a useful reference preparation with which to compare membrane peptidase levels. AP-A, AP-N, CP-M and E-24.11 were fairly abundant in osteoblast-like cell membranes, being approximately 48 %, 5 %, 137 % and 5 % respectively of the specific activities of the renal membranes. The remaining enzyme, AP-W, was present in membrane preparations at only 0.2 % of the activity found in renal membranes.

Immunological evidence for E-24.11 expression on human osteoblast-like cells

Both immunofluorescent staining and e.l.i.s.a. were used to confirm the presence of E-24.11 on osteoblast-like cells. Figure 1 shows immunofluorescent staining for E-24.11 on cells stained before (Figure 1c) and after (Figure 1a) permeabilization with 0.1 % Triton X-100. Before permeabilization there is an overall cell-surface staining of E-24.11. After permeabilization, immunofluorescent staining for E-24.11 shows a similar staining pattern to that obtained when using rhodamine-conjugated lectins (Figure 1b). The lectins concanavalin A and wheat germ agglutinin were used as markers for endoplasmic reticulum and Golgi in permeabilized cells. The staining seen with the lectin conjugates in unpermeabilized cells (Figure 1d) is most likely to be due to the identification by wheat germ agglutinin of *N*-acetylglucosamine sugar residues (Goldstein and Hayes, 1978) on surface proteins. The cell-surface staining seen on intact cells

Table 2 Effect of various inhibitors on calcitonin metabolism by osteoblast-like cell membranes

Incubation of calcitonin (50 μ M) with osteoblast-like cell membranes (2 μ g) for 1080 min was performed in the presence of various inhibitors. The amount of hydrolysis of calcitonin was determined by h.p.l.c. With no inhibitors present the membrane preparation hydrolysed 1.6 pmol of calcitonin/min per mg. Results are the means of duplicate assays which did not differ by more than 5%.

Inhibitor	Calcitonin hydrolysed relative to that in absence of inhibitors (%)
None	100
Dip-F (100 μ M)	103
Enalaprilat (10 μ M)	100
Cilastatin (100 μ M)	102
Phosphoramidon (1 μ M)	46
EDTA (1 mM)	4
Amastatin (1 μ M)	98
Amastatin (100 μ M)	101
Phosphoramidon (1 μ M) + amastatin (1 μ M)	47

Figure 2 Analysis of h.p.l.c. peptide products formed by incubation of calcitonin with purified porcine E-24.11

See the Experimental section for details. Some minor peaks which were eluted very early in the gradient were also observed in the blank samples. The product peaks are numbered in the order of elution. (a) Calcitonin incubated for 1 h with E-24.11 (1 μ g); (b) calcitonin incubated for 1 h with E-24.11 (1 μ g) in the presence of phosphoramidon (10 μ M); CT, calcitonin; P, phosphoramidon.

is less intense than the intracellular staining seen in permeabilized cells, and as a consequence is not detected on micrographs exposed for optimal intracellular staining.

E.l.i.s.a. of osteoblast cell membranes measured the amount of E-24.11 as 255 ng of E-24.11/mg of protein. This compares with 1480 ng of E-24.11/mg of protein in P4 fractions of pig kidney cortex and 33.7 ng of E-24.11/mg of protein in P2 fractions of Caco-2 cells (day 26) (Howell et al., 1991, 1992, respectively).

Figure 3 Metabolism of calcitonin and the production of the first cleavage product by E-24.11

Purified porcine E-24.11 (1 μ g) was incubated with calcitonin (50 μ M) and the amount of calcitonin remaining (●) and the first detectable cleavage product (○) were monitored by h.p.l.c. See the Experimental section for details.

Hydrolysis of calcitonin and PTH by osteoblast-like cell membranes

Calcitonin was hydrolysed by osteoblast-like cell membranes and the effects of various inhibitors on calcitonin hydrolysis are shown in Table 2. Cilastatin (100 μ M), Dip-F (100 μ M) and enalaprilat (10 μ M) had no effect on the extent of hydrolysis, indicating that MDP, DPP IV or ACE were not involved in the hydrolysis of calcitonin by osteoblast-like cell membranes, consistent with our failure to detect MDP, DPP IV and ACE in membrane preparations (see above). Incubations containing phosphoramidon (1 μ M) decreased the hydrolysis of calcitonin by approximately 50%, indicating that E-24.11 plays a role in its hydrolysis. Inclusion of EDTA (1 mM) in incubations abolished the majority (96%) of the calcitonin hydrolysis. This would suggest that the phosphoramidon-insensitive activity responsible for the remaining calcitonin hydrolysis is a metallo-enzyme. The aminopeptidase inhibitor, amastatin, did not affect the amount of calcitonin hydrolysed. It did, however, affect the pattern of the degradation product peaks formed in the absence of other inhibitors (results not shown). When both phosphoramidon (1 μ M) and amastatin (1 μ M) were present, the amount of calcitonin hydrolysed did not differ from incubations containing phosphoramidon only. Taken together, these results suggest that E-24.11 may play a major role in calcitonin hydrolysis by this membrane preparation.

PTH was not degraded following a 20 h incubation with 2 μ g of osteoblast-like cell membranes (results not shown).

Hydrolysis of calcitonin and PTH by purified porcine E-24.11 and AP-N

Figure 2 shows the hydrolysis of calcitonin by 1 μ g of E-24.11. Nine product peaks were resolved following a 1 h incubation with E-24.11. PTH was more resistant to hydrolysis by E-24.11. After a 20 h incubation with E-24.11 only 19% hydrolysis occurred and only three minor resolvable, but unidentified, product peaks were obtained (results not shown).

The time courses of hydrolysis of these two hormones and that of substance P were compared. The pseudo-first order half-life of substance P was 0.5 min in these conditions (1 μ g of E-24.11,

Figure 4 Initial cleavage site by E-24.11 in calcitonin

See the Experimental section for details. The small arrows indicate the potential hydrophobic cleavage sites. The large arrow indicates the initial cleavage site.

Table 3 Effect of modulators on E-24.11 expression by cultured human osteoblast-like cells

See the Experimental section for details. Cells were cultured in the presence of the various compounds for 48 h after which time membrane fractions were prepared and E-24.11 activities determined (E-24.11 activity in control samples 13.31 ± 0.31 nmol/min per mg). Results are the mean $\pm$ S.E.M. of three membrane preparations. * $P < 0.05$ and † $P < 0.01$ (Student's *t*-test). Each experiment was repeated using cells from different donors. Essentially similar results were obtained on each occasion. Data from a representative experiment are presented here.

Compound		E-24.11 specific activity (% of control)
Insulin	(0.1 nM)	102 ± 5
	(1.0 nM)	97 ± 5
Calcitonin	(0.15 nM)	$154 \pm 19^*$
	(1.50 nM)	$160 \pm 15^\dagger$
PTH	(0.2 nM)	104 ± 27
	(2.0 nM)	114 ± 15
PMA	(160 nM)	$86 \pm 4^\dagger$

Figure 5 Effect of $1,25(\text{OH})_2\text{D}_3$ on E-24.11 expression on human osteoblast-like cells

See the Experimental section for details. Cells were cultured in the presence of various concentrations of $1,25(\text{OH})_2\text{D}_3$ for 48 h after which time fresh media containing $1,25(\text{OH})_2\text{D}_3$ was added and incubated for a further 24 h. Membranes were then prepared and E-24.11 activities then determined. Error bars are of the S.E.M. ($n = 3$). * $P < 0.001$ (Student's *t*-test).

37 °C). The half-lives of calcitonin and PTH were 23 min and more than 1440 min respectively. These results indicate that calcitonin is a moderate substrate and parathyroid hormone a very poor substrate for E-24.11.

The h.p.l.c. profiles for the hydrolysis of calcitonin are shown in Figure 2. The initial product (peak 8) was detected at 5 min. The time courses for calcitonin hydrolysis and the appearance of peak 8 are shown in Figure 3. Following the initial rise, the

amount of product then decreased after 120 min, indicating that it is further hydrolysed by E-24.11. The fraction containing this peak was collected and sequenced. This identified the site of attack by E-24.11 on calcitonin as the Lys¹⁸-Phe¹⁹ bond (Figure 4).

No degradation of calcitonin or PTH was obtained following a 20 h incubation with 1 µg of purified AP-N (results not shown).

The effects of modulators on E-24.11 expression

Table 3 shows the effects that various hormones and phorbol 12-myristate-13-acetate (PMA) have on E-24.11 activity on osteoblast-like cells. Treatment with insulin and parathyroid hormone for 48 h had no effect. However, treatment of cells with calcitonin (1.5 nM for 48 h) produced a significant increase (60%) in E-24.11 activity. A small but significant decrease in E-24.11 activity was seen in membrane fractions after PMA treatment for 48 h. Figure 5 shows that incubation of cells for 72 h with $1,25(\text{OH})_2\text{D}_3$ (10^{-8} – 10^{-6} M) also increased E-24.11 activity.

DISCUSSION

Cells cultured from explants of human trabecular bone have been shown to exhibit stable osteoblast-like phenotypic characteristics, including production of type I collagen and osteocalcin, responsiveness to parathyroid hormone and high constitutive alkaline phosphatase activity which is further increased by $1,25(\text{OH})_2\text{D}_3$ stimulation (Beresford et al., 1984; Gallagher et al., 1986).

The identification of E-24.11 on osteoblast-like cells in culture is in agreement with the findings of Blumberg and co-workers (Indig et al., 1990). E-24.11 was quantified by e.l.i.s.a. and compared with levels found in pig kidney cortex. Although it was not as abundant as in kidney, the enzyme represented 0.02% of the protein found in membrane preparations. As well as E-24.11, various exopeptidases were also identified on membrane preparations (AP-A, AP-N, AP-W and CP-M). Taken together, these five membrane peptidases have the potential to degrade extensively a variety of peptides. In preparations of pig renal microvillar membranes E-24.11 is the enzyme that normally initiates hydrolysis (Stephenson and Kenny, 1987a,b).

Some of the possible peptide/protein substrates present in and around the bone matrix include calcitonin, insulin, osteocalcin and PTH. Calcitonin was hydrolysed by osteoblast-like cell membranes, and inhibitor studies revealed that E-24.11 was one of two major peptidases responsible for 54% of the calcitonin hydrolysed. The other enzyme involved in calcitonin hydrolysis was an unidentified metallo-enzyme that was phosphoramidon-insensitive and was responsible for 42% of the calcitonin hydrolysed. Inhibition by amastatin (to which AP-N and AP-W are sensitive) did not affect the amount of calcitonin hydrolysed, indicating that neither AP-N nor AP-W play a role in the initial attack on calcitonin. Calcitonin is disulphide-linked between the N-terminal cysteine residue and Cys⁷, producing a relatively compact N-terminal region, and this may limit the access of aminopeptidases. There are seven potential hydrophobic cleavage sites in calcitonin where E-24.11 may act. The initial site of cleavage by E-24.11 was identified as that between residues Lys¹⁸ and Phe¹⁹. Calcitonin was found to be a moderate substrate for E-24.11, with a half-life of 23 min compared to 0.5 min for substance P under the conditions used. In contrast, the half-life of PTH was in excess of 20 h. This is consistent with the preference of E-24.11 for oligopeptides rather than polypeptides.

When osteoblast-like cells were cultured in the presence of PMA for 48 h a small but significant decrease (15%) in E-24.11 activity was observed. Werb and Clark (1989) reported a 90%

decrease in E-24.11 mRNA levels when human fibroblasts were treated for 48 h with PMA. However, E-24.11 has been shown to have a half-life of 3–4 days in cultured human fibroblasts (Lorkowski et al., 1987). If the turnover is similar in osteoblast-like cells then membrane activity would not be expected to decrease substantially over the time period used here. Treatment of cells with 1.5 nM calcitonin resulted in a 60% increase in E-24.11 activity. This could reflect a feedback mechanism controlling levels at the cell surface. Incubation of cells with $1,25(\text{OH})_2\text{D}_3$ (10^{-8} – 10^{-6} M) also increased E-24.11 activity. In this concentration range $1,25(\text{OH})_2\text{D}_3$ inhibits cell proliferation (Evans et al., 1990), whereas lower concentrations of between 10^{-14} and 10^{-10} M stimulate proliferation.

Of the five membrane peptidases identified here, E-24.11 may have a significant effect on the levels of polypeptide growth factors in the bone matrix. If hydrolysis of calcitonin by osteoblast cells were to occur *in vivo*, then reduced calcitonin levels may increase osteoclastic resorption of Ca^{2+} from the bone matrix. In this way membrane peptidases, in particular E-24.11, may play a role in bone formation and resorption and hence in Ca^{2+} homeostasis.

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