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Human osteoblast-like cells respond not only to the extracellular calcium concentration but also to its changing rate

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Abstract The effect of extracellular calcium on human osteoblast-like cells (HOS) has been demonstrated. An experimental setup was used for applying defined rates of change in the extracellular calcium concentration. The intracellular calcium concentration was monitored using the fluorescence dye fura-2. HOS cells showed qualitatively different responses of the intracellular calcium concentration to changes of the extracellular calcium concentration depending on its changing rate. A small rate caused only a small and slow increase of the intracellular calcium concentration, whereas faster changes are able to cause a rapid transient increase followed by a sustained elevation of the internal calcium level. Surprisingly, both an increasing as well as a decreasing external calcium concentration is able to cause cellular responses. These signals could be reduced by the IP_3-inhibitor neomycin. We propose that the G-protein dependent signalling pathway of HOS cells can not only sense the extracellular calcium concentration but also its time derivative.

Key words Extracellular calcium · Calcium receptor · Osteoblast-like cells · Fura-2 · Bone remodelling

Abbreviations IP_3 inositol 1,4,5-trisphosphate · $[Ca^{2+}]_i$ intracellular calcium concentration · $[Ca^{2+}]_e$ extracellular calcium concentration · *cAMP* adenosine 3′,5′-cyclic monophosphate

Introduction

Recently a calcium-sensing receptor has been found in several cell types, such as parathyroid and kidney cells (Brown et al. 1993; Riccardi et al. 1995). This sensor is obviously involved in the regulation of the serum calcium level. The direct response of this receptor to small variations of the extracellular calcium concentration leads to the activation of a cascade of cellular reactions. Spikes of the intracellular calcium concentration as well as changes in cAMP level and arachidonic acid release (Brown et al. 1993; Ruat et al. 1995; Riccardi et al. 1995; Emanuel et al. 1996) were found. These signals are supposed to influence the production and secretion of calciotropic hormones, maintaining calcium homeostasis in the body (Brown 1991).

Similar receptors seem to exist even in bone cells (Zaidi et al. 1989; Honda et al. 1995; Leis et al. 1994; Kamioka et al. 1995; Gao et al. 1996; Quarles et al. 1997). In osteoblasts, for example, the rise of extracellular calcium possibly stimulates processes of bone formation. In contrast to the calcium receptors of the above mentioned cells, which are monitoring the systemic calcium level in the blood, osteoblasts sense local concentrations, produced by bone resorbing osteoclasts. These concentrations could reach local values of up to 40 mM (Silver et al. 1988). This implies local concentration gradients and probably variations in a short time scale to which the cells could respond.

The process of bone remodelling includes not only the response of osteoblasts to the bone resorbing activity of osteoclasts. One of the most investigated problems is bone adaptation according to mechanical loading. Therefore various hypotheses are proposed which include mechanical, electromechanical and direct electrical action. Osteoblasts are known to be mechanosensitive, i. e. they respond to shear stress and vibrations (Reich et al. 1990; Lee and Wong 1994; Klein-Nulend et al. 1995; Hung et al. 1996). Up to now, however, the molecular basis of these reactions is not known. Is it possible that a connection exists between the mechanoreceptors and the calcium-regulating system and/or the location of the electrochemical processes in the cell membrane. On the other hand it is not known whether receptor and transporter are separate molecules, or whether the transporters themselves are stress-sensitive.

Some of our preliminary experiments indicated that human osteoblast-like cells respond in a qualitatively different way to various kinds of change in the extracellular calcium concentration, especially to the time scale of a sud-

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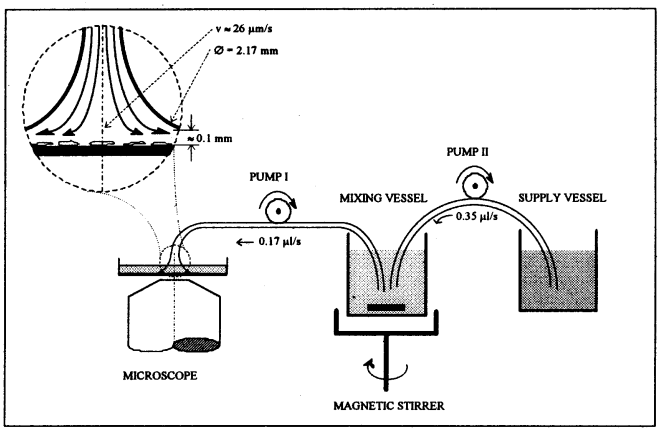

Fig. 1 Scheme of the experimental setup to apply definite rates of change of the extracellular calcium concentration. A funnel shaped opening was positioned over the observed cells and the distance was fixed by small glass pieces. The time rise of calcium concentration in the flow was achieved by a continuous concentration rise in the mixing vessel. Pump I controlled the flow (0.17 µl/s) from the mixing vessel to the coverslip. The rate of concentration rise in this vessel was determined by the concentration of calcium in the supply vessel and/or by pump II

den concentration rise. This effect corresponds to results of other authors (Fried and Tashijan 1986; Nemeth and Scarpa 1987; Zaidi et al. 1989; Brown 1991). Unfortunately, these results could not be compared with each other, because in most cases there was no description of how the concentration change had been done. Therefore we extended the experimental setup to allow us to apply alterations of the calcium concentration near the cell on a well defined time scale. We investigated the influence of various calcium concentrations and gradients on the induction of intracellular calcium signals in single osteosarcoma cells. In this paper we will show that human osteosarcoma cells respond not only to the current concentration of the external calcium, but even to the time dependence of this concentration.

Materials and methods

Materials

The fluorescence dye fura-2 was obtained from Molecular Probes. DMEM : HAM's F12 (1 : 1), amphotericin, penicillin/streptomycin, fetal calf serum (FCS), trypsin/EDTA and Hanks salt solution were from Biochrom. Neomycin and Na_2EDTA were from Sigma. $CaCl_2 \cdot 2H_2O$ were purchased from Fluka. Culture dishes were from Falcon.

Cell culture

Human osteogenic sarcoma cells (HOS, TE-85 from American Type Culture Collection, Rockville, MD) were cultured routinely in DMEM : HAM's-F12 (1 : 1) containing 10% FCS, 1% amphotericin and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2. For every experiment, cells were subcultured and plated on glass coverslips at 10^3 cells/ml and incubated overnight. The coverslips formed the bottom of the experimental chamber for microscopic observations. Its wall consisted of a glass ring (diameter 12 mm, 5 mm high). The use of these thin glass coverslips instead of quartz coverslips did not effect the fluorescence measurements.

Measurement of extracellular calcium by microelectrodes

Microeletrodes with a tip diameter of 20 – 30 µm were prepared and filled with 10 mM $CaCl_2$ and a calcium-ionophore (Cocktail I from Fluka). To measure the real time course of the concentration rise in the funnel which covers the cells (Fig. 1), the tips of the bent electrodes were positioned horizontally under the opening of the funnel.

Measurement of intracellular calcium

The cells (HOS) were loaded with fura-2/AM (2 – 4 µM, 60 min, 37 °C) dissolved in Hanks salt solution containing 1 mM $CaCl_2$ (HSS). After washing, the cells were transferred into HSS. The fluorescence was measured using an inverse fluorescence microscope (Attofluor, Carl Zeiss) equipped for alternate recording at two excitation wavelengths (334/380 nm). In most of the experiments the fluorescence ratio 334/380 nm was taken every 5 seconds. The intracellular calcium concentration was calculated using calibrated concentrations of fura-2 salt. All experiments were performed at 37 °C.

The experimental setup to apply time dependent external calcium concentrations

For our experiments we needed an experimental setup which allows one to measure the fura-2 fluorescence of the cells simultaneously during a controlled alteration of the external calcium concentration. The rate of concentration change should be adjustable independently of the hydrodynamic conditions near the cells. These conditions, contradict each other. On the one hand, minimal shear stress should be applied, protecting the cell from mechanical excitation. On the other hand, it should be possible to modify the calcium concentration near the cell surface quickly and without significant time delay. As a compromise, we used a setup, schematically demonstrated in Fig. 1. A funnel shaped opening was positioned over the microscopically observed cells by a micromanipulator. The distance between the edge of this funnel and the coverslip was fixed by small pieces of a coverslip. An optimal flow rate of 0.17 µl/s was realised by pump I. Therefore the cells were surrounded by a constant laminar flow of saline in all ex-

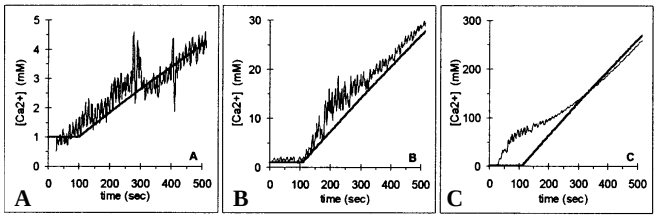

Fig. 2 A–C Measured versus predicted extracellular calcium concentrations near the cells for three different rates of concentration rises (dc/dt). The extracellular calcium concentration was measured using ion-selective microelectrodes. The theoretical concentration rate is dc/dt = 0.008 mM/s (**A**), dc/dt = 0.070 mM/s (**B**), dc/dt = 0.700 mM/s (**C**)

periments, independent of the adjusted rate of concentration change. The time rise in calcium concentration in this flow was achieved by a continuous concentration rise in the mixing vessel. The rate of concentration rise in this vessel was determined by the concentration of calcium in the supply vessel and/or by pump II.

First this setup was used just to increase the calcium concentration continuously near the cells. Later we used the same setup to investigate the diminution of the extracellular calcium concentration. This is possible in the same way be continuously adding EDTA into the mixing vessel.

The calcium concentration in the mixing vessel can be described by the following equations:

$$\frac{dc_2(t)}{dt} + c_2(t)\frac{\dot{V}_{12}}{V(t)} = \frac{\dot{V}_{12}}{V(t)}c_1 \tag{1}$$

$$V(t) = V_{20} + (\dot{V}_{12} - \dot{V}_{23})t \tag{2}$$

where $c_2(t)$ is the current calcium concentration in the mixing vessel, $V(t)$ is the volume of the mixing vessel, c_1 is the calcium concentration in the supply vessel, V_{20} is the volume of the mixing vessel at $t = 0$, $\dot{V}_{12}$ is the volume flow rate from supply to mixing vessel and $\dot{V}_{23}$ from the mixing vessel to the coverslip.

For the condition at $t = 0$, $c_2(t) = c_{20}$, this equation can be solved:

$$c_2(t) = c_1 + (c_{20} - c_1)\left(1 + t\frac{\dot{V}_{12} - \dot{V}_{23}}{V_{20}}\right)^{-\frac{\dot{V}_{12}}{\dot{V}_{12} - \dot{V}_{23}}} \tag{3}$$

The slope in the interesting time interval is assumed to be constant.

These calculations were checked experimentally using ion-selective microelectrodes. Figure 2 demonstrates the measured values and the curve theoretically predicted. It shows that the above description is sufficiently correct for small rates of calcium increase. At higher rates, especially at the beginning, an increased rise of the concentration was measured, which later became flatter and closer to the theoretical curves. Furthermore, we observed concentration changes at the electrodes even before they were theoretically expected. This expectation is based on the dead time of the system, which was difficult to estimate exactly. This dead time was determined experimentally by observing the passage of small air bubbles in the tube. However, in the real case a fast central tongue of the streaming fluid can cause locally increased concentration gradients. Furthermore, the influx of the fluid into the funnel can effect the streaming profile. Thus, the time difference increases with increasing slope of the concentration curves.

Results

Experiments with increasing concentrations of external calcium

Two typical experiments, demonstrating the response of cells to different rates of increasing the external calcium concentration are shown in Fig. 3. Only the fast change of the extracellular calcium concentration was able to cause a rapid overshooting increase of the calcium concentration (spike) in the cell. The increase of intracellular calcium continued even when the external concentration had already reached the plateau. In the case of slower external concentration rises, no intracellular calcium spike could be observed. The internal calcium concentration was simply adjusted to correspond to the new extracellular conditions. Thus, this process took place as long as the external concentration was rising. In all experiments the external calcium concentration rose from 1 mM to a final concentration of 10 mM more or less rapidly. Consequently, the level of the finally reached internal calcium concentration was always the same.

We classified the time course of the intracellular calcium concentration of every single cell either as “slow increase”, as “fast overshooting reaction (spike)” or as “no reaction”. In the latter class we counted all cells whose intracellular calcium concentration rose by less then 25% with respect to the initial value. The dependence of the relative occurrence of intracellular calcium spikes on the rate of concentration changes (d*c*/d*t*) is a sigmoid function (Fig. 4). For these experiments with different changing rates of the extracellular concentration from 1 mM to 10 mM, the critical rate is (0.046±0.015) mM/s. The variations in the experimental results increase with the concentration rate. This can be explained by the deviation between the calculated and the actually measured values of the external concentration rise, as demonstrated in Fig. 2.

Pre-treatment with neomycin

To clarify whether the phosphatidyl inositol turnover contributes to the generation of the intracellular calcium signal, the HOS cells were pre-treated with neomycin (1 μM, 1 h) which inhibits the activity of phospholipase C (Lipsky and Leitman 1982). Neomycin was also present in the measuring buffer. In these experiments the effect of high extracellular calcium concentrations was strongly reduced (Fig. 3 D). Some cells showed a biphasic behaviour

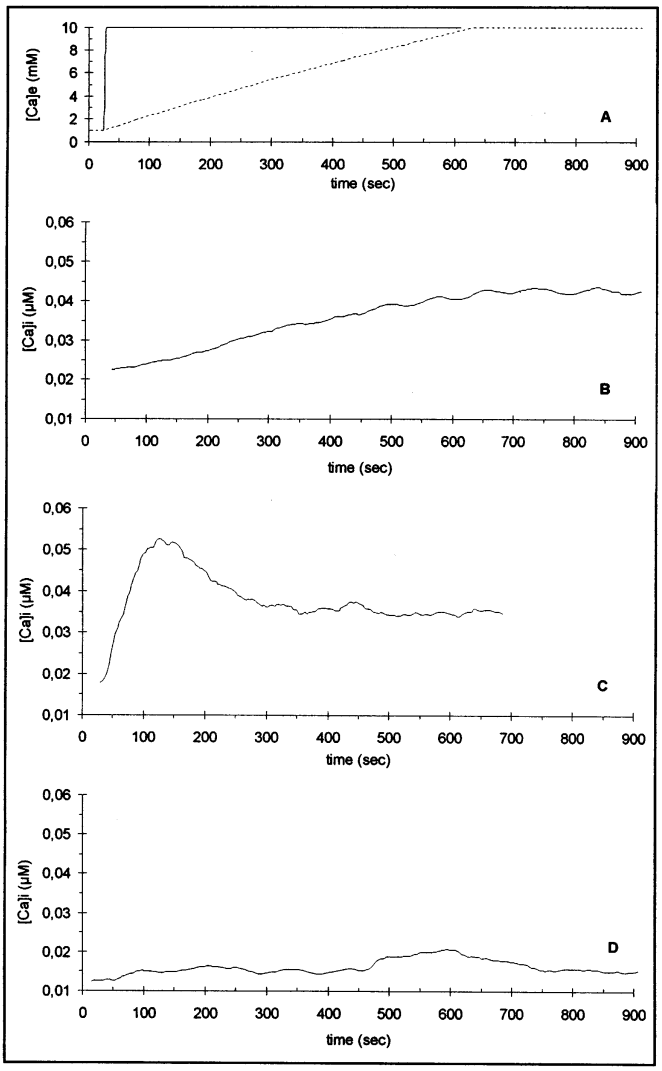

Fig. 3A–D The intracellular calcium concentration in response to different rates of increasing the extracellular calcium concentration. **A** The time course of the extracellular calcium concentration (dc/dt): 1.73 mM/s (*solid line*); 0.03 mM/s (*dotted line*). **B–D** Intracellular calcium concentration in the case of dc/dt = 0.03 mM/s (**B**), dc/dt = 1.73 mM/s (**C**). In **D** the cells were pre-treated with 1 µM neomycin. Neomycin was also present in the measuring buffer; dc/dt = 0.87 mM/s. The curves **B–D** represents mean values of 34 cells

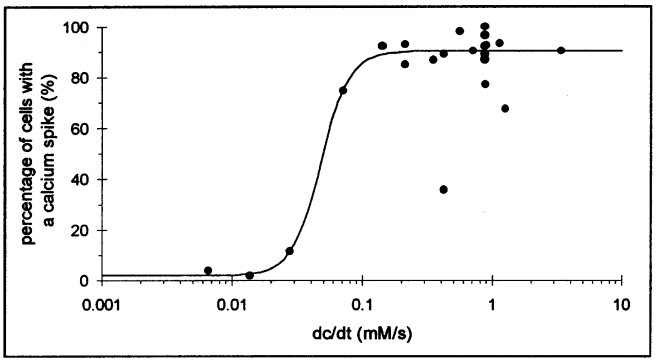

Fig. 4 Percentage of cells with an intracellular calcium spike in relation to varying changing rates of the extracellular calcium concentration. Every point represents results of one independent experiment, including 30–50 cells. The data were fitted ($r^2 = 0.82$) by the sigmoid function: $y = y_{min} + \frac{y_{max} - y_{min}}{1 + \left(\frac{x}{x_{50}}\right)^{-n}}$

in the following sense: they responded to increased extracellular calcium with a slight increase of intracellular calcium (see Fig. 3D), but after ten minutes a calcium spike appeared suddenly.

Furthermore it is known, that neomycin is able to act as a stimulus itself in causing an intracellular calcium increase. This, however, did not influence our measurements because neomycin had already been added 1 h before the calcium measurement started and it was present during the whole experiment. On the other hand we could show that after a sufficient relaxation period cells could respond several times to a stimulus with a calcium spike. Furthermore, the cells did not show an anomalous behaviour in these experiments. So, the cells should have been able to respond to external calcium concentrations, even if neomycin has caused a calcium signal.

Experiments with decreasing concentrations of external calcium

If the observed cellular reactions on increasing extracellular calcium are based on a receptor–mediated process, then similar behaviour is also expected in the case of decreasing concentrations. Sufficiently fast changes of the concentration of free calcium were obtained by continuously adding EDTA to the external solution.

We found that even a decreasing extracellular calcium concentration affects the intracellular calcium concentration (Fig. 5). Again there were two qualitatively distinct reactions depending on the rate of concentration change: A fast change caused a transient *increase* of the intracellular calcium concentration, which is sometimes followed by a slower, sustained increase. Slower changes of the external concentration again caused a simple adjustment of the intracellular calcium concentration to the extracellular concentration.

Discussion

An increase of the intracellular calcium concentration as a response to high extracellular calcium concentrations has already been found for cells of parathyreoidea (Brown 1991), kidney (Riccardi et al. 1995), brain (Ruat et al. 1995; Emanuel et al. 1996), placenta (Juhlin et al. 1990), and for keratinocytes (Reiss et al. 1991), osteoclasts (Zaidi et al. 1989) and osteocytes (Kamioka et al. 1994). Brown et al. (1993) isolated and cloned a calcium sensitive receptor of parathyroid cells and identified its primary structure. Thus, at least in these cells the response to extracellular

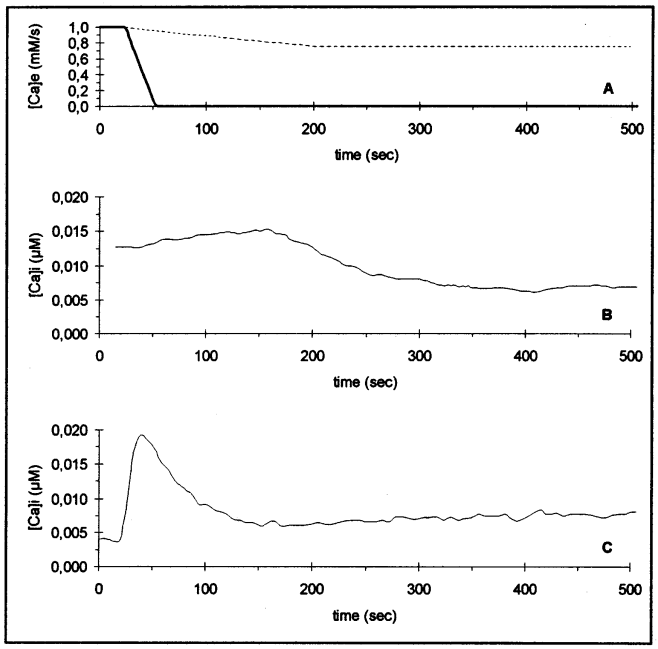

Fig. 5 A–C The intracellular calcium concentration in response to different rates of decreasing the extracellular calcium concentration. **A** The time course of the extracellular concentrations (dc/dt): – 0.035 mM/s (*smooth line*); – 0.002 mM/s (*dotted line*). **B, C** Intracellular calcium concentration in the case of dc/dt = –0.002 (**B**), and dc/dt = –0.035 mM/s (**C**). The curves **B, C** represents mean values of 32 cells

calcium was found not simply to be caused by a calcium channel, but rather by a special receptor molecule.

Leis et al. (1994) found a corresponding behaviour in murine osteoblast-like cells. Our own experiments confirm these results. We found that human osteoblast-like cells increase their internal calcium concentration as a result of rapidly increased as well as decreased external concentration. There, even very small concentration changes are effective. This indicates that it is not simply a process of a small change of the 10^4:1 relation of external to internal concentration, but rather a result of a signal transformation. There are two hypothetical possibilities describing the behaviour of the cells. Both have to include dynamic properties. First an ion channel could act, which is coupled in some way to a calcium sensor. This protein could control the calcium influx according to the existing concentration gradient. In the following a calcium induced calcium release can lead to intracellular calcium spikes. A slow concentration change gives the cells more time to handle this, so no spike is observed. However, decreasing the external concentration nearly down to zero should prevent an internal calcium increase. Contradicting this expectation we again found a calcium spike (Fig. 5C), forcing us to reject the idea of a receptor coupled ion channel. These signals particularly suggest the intracellular origin of the involved calcium.

The second hypothesis postulates a special calcium receptor, which can release calcium from intracellular stores via the formation of second messengers. That model permits an internal calcium spike for both cases, increasing as well as decreasing external concentration. It is supported by the experiments with neomycin (Fig. 3D), indicating that IP_3 is involved in the generation of the observed calcium signals. Furthermore, we found that human osteosarcoma cells respond not only to the external calcium concentration (c_e), but, additionally, to its time derivative (dc_e/dt), i.e. to the rate of external concentration change. To investigate this cellular response, we used an experimental setup which allowed us to change this time derivative, without changing the hydrodynamic conditions near the cells (Fig. 1). However, this setup fulfils these conditions only approximately. The comparison of the measured rates of calcium concentration changes near the measured cells with the calculated curves, shows some deviations (Fig. 2) for fast concentration changes.

The main questions are: how do these deviations modify our results, and: how one could avoid these difficulties? In general, the flow in the tube and in the funnel of this system can be characterised by very small Reynolds numbers. Therefore it is supposed to be laminar. A parabolic velocity profile in the tube, however, causes (in the case of increasing concentrations in the mixing vessel) the concentration near the centre of the tube to be higher than in the periphery. Furthermore, reflections at the coverslip may produce additional heterogeneities. These deviations increase with increasing rates of concentration changes. This weakness of the experimental setup could have been avoided only by slowing down the rate of concentration change. This, however, would not bring us to the region of interest. Therefore our results can only qualitatively indicate the response of the cells to the rate of calcium concentration changes.

The experiments which were done with neomycin pretreatment indicate that the IP_3 signalling pathway is at least partly involved. The above mentioned biphasic behaviour of some cells suggests that a further mechanism could exist for increasing the intracellular calcium concentration. For instance a slow calcium influx could lead to a calcium induced calcium release (CICR).

Reiss et al. (1991) found that in the case of high extracellular calcium concentrations the calcium efflux as well as the influx of keratinocytes is increased. We suppose that these transports are very important to establish an intracellular steady state concentration of calcium. In our experiments the adjustment of the intracellular calcium concentration to a slowly changing extracellular concentration demonstrates this fact. Furthermore, the finally reached intracellular concentration is nearly the same in all our experiments done with different concentration rates but with equal final concentrations.

Consequently, at least two different processes of calcium control are going on in the cell, which overlap each other in their action. First, there is a slow adjustment of the cellular calcium level, which is realised by transport mechanisms localised in the cell membrane. Second, a rapid IP_3 mediated process exists, which leads to calcium release from intracellular stores. In contrast to the first process, the second one is not only influenced by concentrations but also by the rate of change of the extracellular calcium con-

centration. It is stimulated only in the case of sufficiently fast changing rates, independent of their direction.

We suggest that there could be a G-protein coupled calcium sensing receptor in the osteoblast-like cell line HOS, similar to that found in other cells. It could be responsible for this IP_3 mediated process. This assumption is supported by Gao et al. (1996) who has detected mRNA of the parathyroid calcium receptor in some human osteoblastic cell lines. It should not be identical to the already cloned and characterised calcium receptor of parathyroid and kidney cells. The biophysical mechanism, explaining the perception of the time derivative is still not known. One can imagine a twofold calcium dependent response to the extracellular stimulus where one way leads to some kind of activation and the other, with a time delay, to an inactivation of processes which cause an increase of intracellular calcium. Molecular investigations are needed to clarify this question.

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