ELSEVIER

Biochimica et Biophysica Acta 1373 (1998) 341–346

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Modulation of rat incisor odontoblast plasma membrane-associated Ca^{2+} with nifedipine

Ted Lundgren *, Anders Linde

Department of Oral Biochemistry, Faculty of Odontology, Göteborg University, P.O. Box 451, S-405 30 Göteborg, Sweden

Received 28 April 1998; revised 29 June 1998; accepted 2 July 1998

Abstract

In addition to the Ca^{2+} portion freely dissociated in the cytosol, another Ca^{2+} pool is associated with plasma membranes and intracellular organelle membranes. This Ca^{2+} portion is of importance for regulation of, among other things, the cell cycle, actin-mediated processes, and cell morphology. In the literature, dihydropyridines have been reported to influence this membrane-associated pool of Ca^{2+} under certain conditions. The aim of this investigation was to study possible modulations of plasma membrane-associated Ca^{2+} upon treatment with nifedipine in vitro in a Ca^{2+}-transporting cell, the dentin-forming odontoblast. The membrane-associated portion of Ca^{2+} in dissected dentinogenically active rat incisor odontoblasts was monitored by fluorescence spectrophotometry using chlortetracycline as a probe. In addition, images of chlortetracycline–Ca^{2+} binding were obtained by fluorescence microscopy. It was found that membrane-associated Ca^{2+} decreased by the dihydropyridine nifedipine, whereas this Ca^{2+} pool was unaffected by the cellular polarization state, which was in contrast to cytosolic free Ca^{2+} as measured by fura-2. The results show that the odontoblast plasma membrane-associated Ca^{2+}-pool can be modulated by nifedipine, thus being dependent on the conformational state of the L-type Ca^{2+} channels.

Keywords: Calcium channel; Chlortetracycline; Dentinogenesis; Dihydropyridine; Nifedipine; Odontoblast

1. Introduction

Odontoblasts play a central role during the formation of dentin in that they synthesize the collagen and non-collagenous macromolecules of the dentin organic matrix [1,2]. In addition, the odontoblasts are instrumental in mineral formation [3]. Earlier evidence shows that a transcellular route for Ca^{2+} transport is of a central importance during active dentinogenesis [3–5]. For this ion transport, a set of specialized mechanisms exist in odontoblasts, which by a concerted action gates Ca^{2+} into the proximal end of the cells and extrude the ions at their distal end, close to the site of mineral formation. As in osteoblasts, a cell type in many respects closely related to odontoblasts, the presence of voltage-gated, dihydropyridine-sensitive Ca^{2+} uptake channels has been described in odontoblasts [6,7]. Inhibition of such channels in vivo has been shown to suppress the uptake of Ca^{2+} ions into the dentin mineral phase during dentinogenesis [8]. In vitro, L-type Ca^{2+} channels in odontoblasts can be manipulated

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium activity; CTC, chlortetracycline hydrochloride; DMSO, dimethylsulfoxide; EGTA, ethylene glycol-*O,O'*-bis(2-aminoethyl)-*N,N,N',N'*-tetra-acetic acid

* Corresponding author. Fax: +46-31-418122;
E-mail: lundgren@odontologi.gu.se

0005-2736/98/$ – see front matter
PII: S0005-2736(98)00124-2

by inhibitory dihydropyridines, such as nifedipine [6,7].

Cellular Ca^{2+} is either freely dissociated in the cytosol or bound to Ca^{2+}-complexing agents such as, among other things, anionic counter ions, proteins, or membranes. Membrane-associated Ca^{2+} is believed to constitute an easily available pool of Ca^{2+} for a variety of cellular functions. In different cell types, the cell cycle, actin-mediated processes, and cell morphology have been reported to be partially dependent on membrane-associated Ca^{2+} [9–13]. During pathological conditions, such as ischemia, anti-tubulin drug treatment, or hypertension, membrane-associated Ca^{2+} may be released [14–18].

Dihydropyridines affect Ca^{2+}-channels of the L-type in plasma membranes [19,20], but have also been reported to, directly or indirectly, influence the membrane-associated pool of Ca^{2+} under certain conditions. In neutrophils, for example, calcium channel antagonists have been shown to inhibit mobilization of membrane-associated Ca^{2+} [21].

In addition to fura-2 and other fluorochromes, chlortetracycline (CTC) has been used for detection of cellular Ca^{2+} by means of both conventional fluorescence spectrophotometry and fluorescence imaging [22–28]. Upon binding to biological membranes, the Ca^{2+}–CTC complex becomes fluorescent, thereby making it a convenient probe for membrane-associated Ca^{2+} [29–31]. In addition, this probe can be used to detect sequestered intracellular Ca^{2+} [22,30,32–36].

The aim of the present study was to discriminate between the membrane-associated and free pools of Ca^{2+}, and to examine whether plasma membrane-associated Ca^{2+}, in addition to free Ca^{2+}, could be modulated by nifedipine in dentinogenically active rat incisor odontoblasts, a highly polarized cell type involved in bulk transport of Ca^{2+} during biomineralization.

2. Materials and methods

Sprague–Dawley rats (body weight 200–400 g; B&K Universal, Sollentuna, Sweden) were used. The rats were conditioned in the animal facility prior to the experiments and fed a conventional diet and water ad libitum. Pluronic F-127 and cyclopiazonic acid were purchased from Calbiochem (La Jolla, CA, USA). DMSO stock solutions of pluronic F-127 (10%) and cyclopiazonic acid (3 mM) were prepared. Digitonin, Triton X-100 and nifedipine were obtained from Sigma (St. Louis, MO, USA). A stock solution of nifedipine (1 mM) in DMSO was prepared. Fura-2/AM and chlortetracycline hydrochloride were acquired from Fluka (Buchs, Switzerland). A stock solution of fura-2/AM (5 mM) in DMSO was prepared. The nifedipine, CTC and fura-2 stocks were stored in light-safe boxes, and light exposure, except during fluorescence measurements, was avoided. DMSO was purchased from Merck (Darmstadt, Germany). All other reagents were of the highest purity available.

2.1. CTC fluorescence spectroscopy

For each measurement, odontoblasts from two rats were dissected out [37]. Cellular CTC loading was in essence accomplished as described by Jy and Haynes [34]. The cells obtained were preincubated at 6°C in a CTC-containing (10 μM), calcium- and magnesium-free Ringer medium for 30 min, after which an additional incubation was performed for 20 min in a thermostated cuvette at 37°C. When nifedipine was present, it was used at a 5 μM concentration. Every single addition (30 μl) to the cuvette corresponded to 1% of the total sample volume (3 ml). Cellular calcium influx was initiated by the addition of $CaCl_2$ at a 2 mM final concentration. CTC measurements were performed with a Perkin–Elmer LS 50B luminescence spectrometer at 390 nm excitation and 515 nm emission wavelengths in a quartz cuvette at 37°C. The arbitrary fluorescence units were set to zero at the onset of measurement. The integrity of the cells was controlled at the end of each measurement by the addition of digitonin to the cuvettes to check for alterations in fluorescence.

2.2. CTC fluorescence microscopy

CTC incubations were performed essentially according to the method of Engelmann et al. [22]. Dissected odontoblasts [37] from two rats, i.e., eight incisors, were Ca^{2+} depleted in a cyclopiazonic acid (30 μM)- and EGTA (0.1 mM)-containing medium. After 1 h of incubation at 37°C, cells were collected

at 500×*g* for 10 min and washed four times in the buffer prior to the CTC incubation. The odontoblasts were then incubated in a Ringer medium containing 100 μM CTC at 37°C for 30 min, and the cells were pelleted at 500×*g* for 10 min and washed once. The cells were resuspended in 50–100 μl of buffer and were placed on a glass slide with a glass cover. Fluorescence was recorded at room temperature in a Nikon Diaphot fluorescence microscope equipped with a BV-2A fluorescence filter block.

2.3. Fura-2 fluorescence spectroscopy

For each measurement, odontoblasts from incisors of 4–5 rats were dissected out [37]. Fura-2/AM loading was performed essentially as described by Thomas and Delaville [38]. Briefly, freshly dissected cells were incubated for 30 min in a fura-2/AM (5 μM)- and Pluronic F-127 (0.025%)-containing Ringer medium at 37°C with slow stirring. The cells were spun down at 500×*g* for 10 min and washed twice. The cell pellet was then suspended in the Ringer medium, being either calcium-containing or not. Nifedipine, if present, was used at a 5 μM concentration. In some measurements, cellular calcium influx was initiated by the addition of $CaCl_2$ at a 2 mM final concentration. At the end of each run, fluorometry calibrations were made with additions of Triton X-100 followed by EGTA. Every single addition of drugs or chemicals to the measurement cell corresponded to 1% (30 μl) of the total sample volume (3 ml). Intracellular calcium measurements were performed in a double-beam luminescence spectrometer at 340 and 380 nm excitation and 510 nm emission wavelengths, in a quartz cuvette at 37°C with slow magnetic stirring. $[Ca^{2+}]_i$ was calculated using $K_d = 220$ for fura-2.

2.4. Plasma membrane depolarization

In one series of experiments, odontoblast plasma membranes were depolarized during the measurements. This was achieved by replacing the Ringer medium with a high K^+-containing medium (120 mM). In the CTC measurements, the cells were spun down as above after the CTC incubation and afterwards resuspended in the high-K^+ medium. In the fura-2 measurements, the high-K^+ medium was added after the last pellet wash.

Results are presented as means ± S.E.M. Statistical analyses were performed using a computerized paired Student's *t*-test (StatView 4.01). The experiments were reviewed and approved by the Animal Research Ethics Committee at Göteborg University.

3. Results

3.1. CTC fluorescence spectroscopy

When treated with nifedipine in a physiological, low-K^+ medium, the CTC fluorescence was found to decrease, reaching its lowest level after 600 s (Fig. 1). This decrease differed significantly from the baseline level ($P < 0.001$, $n = 6$).

The CTC fluorescence was not altered when odontoblasts in a Ca^{2+}-containing medium were depolarized by a 120 mM K^+-containing medium. The addition of nifedipine under these conditions did not result in any statistically significant difference in fluorescence either (Fig. 1).

In the fluorescence microscope, the circumference of CTC-loaded odontoblasts was clearly delineated. The periphery of the nucleus as well as non-discernible intracellular structures were also vaguely labeled

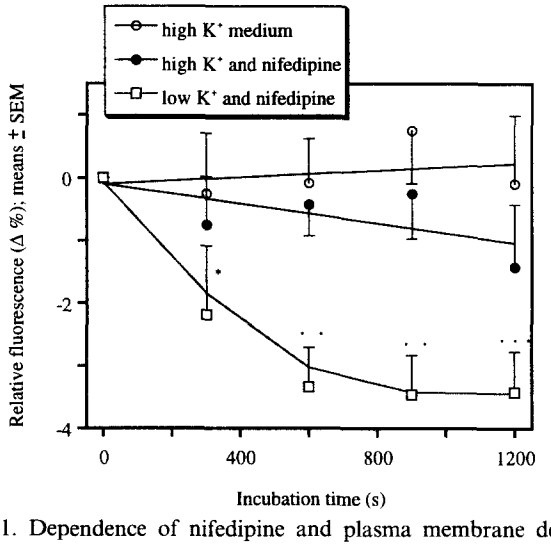

Fig. 1. Dependence of nifedipine and plasma membrane depolarization on CTC fluorescence. The nifedipine effects differed significantly from the baseline value ($P < 0.001$, n=7). When depolarized in a K^+-containing medium (120 mM), the odontoblast CTC fluorescence in a Ca^{2+}-containing medium did not change. A further addition of nifedipine did not yield any statistically significant effect on the fluorescence ($n = 7$).

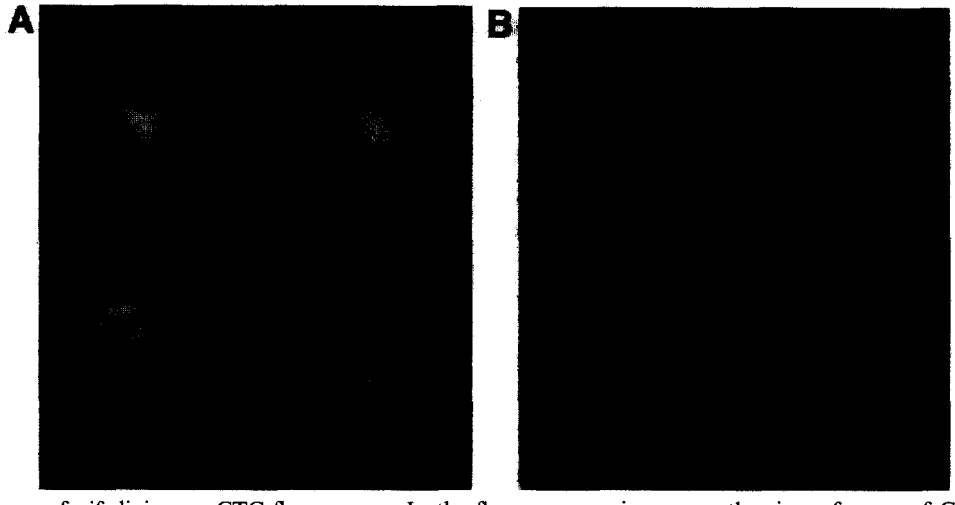

Fig. 2. (A) Dependence of nifedipine on CTC fluorescence. In the fluorescence microscope, the circumference of CTC-treated odontoblasts appeared clearly. The periphery of the nucleus and non-discernible intracellular structures were also vaguely labeled. (B) After treatment with nifedipine, the cell surface CTC fluorescence disappeared, in addition to the intracellular labeling. Bars = 50 μm.

(Fig. 2A). After treatment with nifedipine, the plasma membrane CTC fluorescence disappeared as did the other intracellular labeling (Fig. 2B).

3.2. *Fura-2 fluorescence spectroscopy*

When depolarized in a K^+-containing medium (120 mM), odontoblasts exhibited a rise in $[Ca^{2+}]_i$ upon addition of extracellular Ca^{2+}, reaching a maximum value after 300 s (Fig. 3). In contrast, this effect was not obtained in the presence of nifedipine, and this difference was significant after 300 s ($P < 0.05$, $n = 6$).

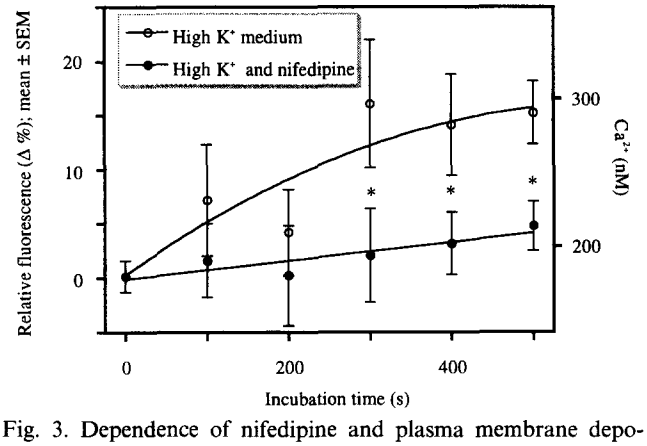

Fig. 3. Dependence of nifedipine and plasma membrane depolarization on fura-2 fluorescence. When depolarized in a K^+-containing medium (120 mM), the cells exhibited an increase in $[Ca^{2+}]_i$ upon addition of extracellular Ca^{2+}, reaching a maximum value after 300 s. Under the same conditions, nifedipine-treated cells differed significantly from the untreated cells after 300 s ($P < 0.05$, $n = 6$).

4. Discussion

Ca^{2+} binding, influx and release were measured with fluorescence spectroscopy using CTC and fura-2 as Ca^{2+} probes [29–31,38]. CTC has been used for detection of cellular Ca^{2+} by means of both conventional fluorescence spectrophotometry, and fluorescence imaging. In contrast to CTC, fura-2 reflects cytosolic free Ca^{2+} [38], whereas CTC measures membrane-associated Ca^{2+}. Due to its low dissociation constant for Ca^{2+}, fura-2 is convenient for the detection of Ca^{2+} within the nanomolar range. The apparent K_d of CTC for Ca^{2+} in an aqueous environment is about 400 μM [39,40] or even as high as about 3 mM [25]. Due to this high K_d for Ca^{2+}, the CTC fluorescence signal originates from pools in cells with mM concentrations of Ca^{2+}. CTC has, among other things, been used to evaluate the acrosome reaction of sperm [27,28], as well as intravesicular Ca^{2+} in a variety of cells [22–25,29,33,39,40]. The probe has, however, so far never been used to detect cellular Ca^{2+} in a cell type involved in biomineralization. Other types of tetracyclines, though, have been shown to interact

with osteoblasts. Chemically modified tetracycline binds along plasma membranes of osteoblasts [26]. In an aqueous milieu, CTC binds to Ca^{2+} in proportion to the $[Ca^{2+}]_i$ [33], and then preferentially associates with membranes [29,34,41–44]. Alterations in fluorescence represent changes in the pool of membrane-associated Ca^{2+} [43], where fluorescence intensity increases about 20-fold upon Ca^{2+}-binding [25].

When incubating odontoblasts with cyclopiazonic acid, a Ca^{2+}-ATPase inhibitor, the endoplasmic reticulum Ca^{2+}-ATPase is impaired, resulting in a Ca^{2+} release from the endoplasmic reticulum and an increase in $[Ca^{2+}]_i$ [7]. A simultaneous treatment with EGTA withdraws Ca^{2+} that can bind to CTC. This procedure was performed prior to treatment with CTC and extracellular Ca^{2+} in order to extinct intracellular fluorescence. Thus, the fluorescence observed primarily emanated from CTC–Ca^{2+} complexes bound to the exterior plasma membrane.

The cell cycle, actin-mediated processes, and cell morphology have been shown to be, at least in part, dependent on membrane-associated Ca^{2+} [9–13]. Further, during ischemia, anti-tubulin drug treatment or hypertension, membrane-associated Ca^{2+} may be released [14–18]. This membrane-associated pool of Ca^{2+} has been reported to be sensitive for dihydropyridines; in neutrophils, for example, calcium channel antagonists inhibit mobilization of this Ca^{2+} pool [21]. Upon binding of nifedipine, the amount of membrane-associated Ca^{2+} in odontoblasts decreased. If the plasma membranes were depolarized, the CTC fluorescence was not influenced by the presence of nifedipine. (Fig. 1). In the depolarized, open state, the Ca^{2+}-channel Ca^{2+} affinity was not affected by the inhibitory nifedipine. The changes in amount of membrane-associated Ca^{2+} after treatment with nifedipine may be interpreted as alterations in the membrane affinity for Ca^{2+}. When in a closed conformational state, the L-type Ca^{2+}-channel affinity for Ca^{2+} might be changed. The possibility of nifedipine to, in addition, influence membrane-associated Ca^{2+} not in conjunction with Ca^{2+}-channels cannot be ruled out, however. Nifedipine might, for example, influence the lipophilic properties of the CTC–Ca^{2+} complexes, or in some other way quench the CTC–Ca^{2+} complex fluorescence at the dose (5 μM) used. It might also be possible that part of the fluorescence decrease is due to nifedipine making less Ca^{2+} available to intracellular, and fluorescent, Ca^{2+} stores.

In the fluorescence microscope, the odontoblast plasma membrane-associated CTC fluorescence appeared clearly (Fig. 2A). After treatment with nifedipine the cell surface CTC fluorescence disappeared in addition to the intracellular labeling (Fig. 2B). This result shows that it is possible to deplete odontoblasts of Ca^{2+} with cyclopiazonic acid and EGTA, and that the CTC fluorescence observed was a nifedipine-sensitive binding of Ca^{2+} to the plasma membrane, in concordance with the fluorescence spectrophotometry measurements.

In fura-2 fluorescence measurements, depolarization increased the $[Ca^{2+}]_i$ in a Ca^{2+}-containing medium, demonstrating the opening of voltage-gated Ca^{2+} channels (Fig. 3). This is in accordance with our earlier findings [8], where nifedipine exhibited a pronounced effect on Ca^{2+} uptake into dentin in vivo. The functional importance of the L-type Ca^{2+} channels during osteogenesis and dentinogenesis is shown by the fact that their specific inhibition in vivo strongly suppresses the uptake of Ca^{2+} ions into both dentin and bone [8,45]. The lack of a total inhibition of inward Ca^{2+} fluxes, observed upon nifedipine treatment, is most probably explained by the presence of other Ca^{2+} uptake pathways as well [7].

Nifedipine decreased the cell surface affinity properties for Ca^{2+}, in addition to inhibition of cellular Ca^{2+} uptake. In the depolarized open state, the Ca^{2+} channel Ca^{2+} affinity was not affected by the inhibitory nifedipine. Ca^{2+} uptake, with an increase in $[Ca^{2+}]_i$, however, was stimulated by depolarization. In addition to further evidence for the presence of voltage-gated, dihydropyridine-sensitive Ca^{2+} channels in rat incisor odontoblast plasma membranes, this in vitro study demonstrated a modulation by nifedipine of the plasma membrane-associated pool of Ca^{2+}. The membrane affinity for Ca^{2+} might in part be dependent on the conformational state of, presumably, L-type Ca^{2+} channels. As these channels are instrumental for hard tissue formation [8,45], a dihydropyridine-dependent membrane affinity for Ca^{2+} might be a factor of significance for biological mineralization.

Acknowledgements

This study was supported by the Faculty of Odontology, University of Göteborg, and the Swedish Medical Research Council (Grants 2789 and 10589).

References

[1] A. Linde, Anat. Rec. 224 (1989) 154–166.
[2] A. Linde, M. Goldberg, Crit. Rev. Oral Biol. Med. 4 (1993) 679–728.
[3] A. Linde, T. Lundgren, Int. J. Dev. Biol. 39 (1995) 213–222.
[4] J.W. Bawden, Anat. Rec. 224 (1989) 226–233.
[5] T. Lundgren, U. Nannmark, A. Linde, Calcif. Tissue Int. 50 (1992) 134–136.
[6] D. Seux, A. Joffre, M. Fosset, H. Magloire, Arch. Oral Biol. 39 (1994) 167–170.
[7] T. Lundgren, A. Linde, Calcif. Tissue Int. 60 (1997) 79–85.
[8] T. Lundgren, A. Linde, Bone Miner. 19 (1992) 31–44.
[9] T. Bengtsson, Exp. Cell Res. 191 (1990) 57–63.
[10] W.C. Hulsmann, L.E. De Wit, C. Schneydenberg, A.J. Verkleij, Biochim. Biophys. Acta 1033 (1990) 214–218.
[11] K. Ikeda, T. Takasaka, Hear. Res. 66 (1993) 169–176.
[12] K. Ikeda, T. Takasaka, Jpn. J. Physiol. 43, (Suppl. 1) (1993) S171–173.
[13] S. Saran, M. Azhar, P.S. Manogaran, G. Pande, V. Nanjundiah, Differentiation 57 (1994) 163–169.
[14] D.R. Eisenmann, A.H. Salama, A.M. Zaki, S.H. Ashrafi, J. Histochem. Cytochem. 38 (1990) 1469–1478.
[15] D.R. Eisenmann, A.H. Salama, A.M. Zaki, J. Histochem. Cytochem. 40 (1992) 143–151.
[16] Y. Hojo, H. Ebata, U. Ikeda, Y. Tsuruya, T. Natsume, K. Shimada, J. Hypertens. 10 (1992) 513–520.
[17] M.O. Samoilov, D.G. Semenov, E.I. Tulkova, J.W. Lazarewicz, Resuscitation 23 (1992) 33–43.
[18] E. Jimenez, P. Del Nido, H. Feinberg, S. Levitsky, J. Thorac. Cardiovasc. Surg. 105 (1993) 988–994.
[19] R.W. Tsien, D. Lipscombe, D.V. Madison, K.R. Bley, A.P. Fox, Trends Neurosci. 11 (1988) 431–438.
[20] H.G. Knaus, T. Moshammer, K. Friedrich, H.C. Kang, R.P. Haugland, H. Glossman, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 3586–3590.
[21] K. Irita, I. Fujita, K. Takeshige, S. Minakami, J. Yoshitake, Biochem. Pharmacol. 35 (1986) 3465–3471.
[22] B. Engelmann, U. Schumacher, J. Duhm, J. Cell Physiol. 143 (1990) 357–363.
[23] J. Tao, D.H. Haynes, J. Biol. Chem. 267 (1992) 24972–24982.
[24] J. Tao, J.S. Johansson, D.H. Haynes, Biochim. Biophys. Acta 1105 (1992) 29–39.
[25] D.C. Renard-Rooney, G. Hajnoczky, M.B. Seitz, T.G. Schneider, A.P. Thomas, J. Biol. Chem. 268 (1993) 23601–23610.
[26] T. Sasaki, N.S. Ramamurthy, L.M. Golub, Bone 15 (1994) 373–375.
[27] L.R. Fraser, L.R. Abeydeera, K. Niwa, Mol. Reprod. Dev. 40 (1995) 233–241.
[28] W.H. Wang, L.R. Abeydeera, L.R. Fraser, K. Niwa, J. Reprod. Fertil. 104 (1995) 305–313.
[29] D.E. Chandler, J.A. Williams, Nature 268 (1977) 659–660.
[30] A.H. Caswell, Int. Rev. Cytol. 56 (1979) 145–181.
[31] A.S. Schneider, R. Herz, M. Sonenberg, Biochemistry 22 (1983) 1680–1686.
[32] D.E. Chandler, J.A. Williams, J. Cell Biol. 76 (1978) 386–399.
[33] D. Dixon, N. Brandt, D.H. Haynes, J. Biol. Chem. 259 (1984) 13737–13741.
[34] W. Jy, D.H. Haynes, Circ. Res. 55 (1984) 595–608.
[35] E. Rubin, R.A. Schlegel, P. Williamson, J. Cell Physiol. 126 (1986) 53–59.
[36] T.D. Coates, M. Torres, J. Harman, V. Williams, Blood 69 (1987) 1146–1152.
[37] A. Linde, Arch. Oral Biol. 17 (1972) 1209–1212.
[38] Thomas, A.P., Delaville, F., in: J.G. McCormack, P.H. Cobbold (Eds.), Cellular Calcium. A Practical Approach, Oxford University Press, Oxford/New York, 1991, pp. 1–54.
[39] A. Fabiato, F. Fabiato, Nature 281 (1979) 146–148.
[40] R.N. Kolesnick, M.C. Gershengorn, J. Biol. Chem. 260 (1985) 707–713.
[41] A.H. Caswell, J.D. Hutchison, Biochem. Biophys. Res. Commun. 42 (1971) 43–49.
[42] A.H. Caswell, J.D. Hutchison, Biochem. Biophys. Res. Commun. 43 (1971) 625–630.
[43] J.E. Smolen, G. Weissmann, Biochim. Biophys. Acta 720 (1982) 172–180.
[44] J.R. Blinks, W. Gil Wier, P. Hess, F.G. Prendergast, Prog. Biophys. Mol. Biol. 40 (1982) 69–72.
[45] J. Duriez, B. Flautre, M.C. Blary, P. Hardouin, Calcif. Tissue Int. 52 (1993) 120–124.