Characterization of Calcium Translocation across the Plasma Membrane of Primary Osteoblasts Using a Lipophilic Calcium-sensitive Fluorescent Dye, Calcium Green C_{18}^{*}

(Qin P. Lloyd†, Michael A. Kuhn‡, and Carol V. Gay¶)

From the†Department of Biochemistry and Molecular Biology and the‡Department of Poultry Science, The Pennsylvania State University, University Park, Pennsylvania 16802 and §Molecular Probes, Inc., Eugene, Oregon 97402

The synthesis of Calcium Green C_{18}, a lipophilic fluorescent calcium-sensitive dye, and its use as a monitor of Ca^{2+} efflux from cells is described. This indicator consists of a Calcium Green-1 molecule conjugated to a lipophilic 18-carbon alkyl chain which will intercalate into cell membranes. The \( K_d \) of the indicator for Ca^{2+} in aqueous solution (pH 7.2, 22 °C, ionic strength 0.1 M) is 0.23 ± 0.04 \( \mu \)M and in the presence of liposomes is 0.062 ± 0.007 \( \mu \)M. Due to its high negativity, the calcium chelating fluorophore faces the cell exterior, when loaded under a defined set of conditions. The dye was found largely on the surface of the cells when loaded at a concentration of 5 \( \mu \)M for 10 min at 37 °C. Five minutes after introduction of EGTA, 83-95% fluorescence disappeared, indicating that most of the fluorophore was on the cell surface. Photobleaching was minimal (3-13%). A confocal laser scanning microscope was used to detect and quantify fluorescence. Internalized dye was apparent in cells loaded for longer times (30-60 min) and in membrane-impaired cells, as shown by uptake of propidium iodide. Under defined confocal laser scanning microscope settings, a transient fluorescence at the periphery of ~30% of the cells was observed following 10^{-8} M parathyroid hormone treatment, indicating the presence of outwardly directed calcium transport across the plasma membrane. Calcium efflux usually lasted 7-10 min, peaking at around 2-3 min. Changes in cell shape were also observed. Calcium efflux was shown to be sensitive to (a) 10 \( \mu \)M quercetin and 10 \( \mu \)M vanadate, partially specific inhibitors of plasma membrane Ca^{2+}-ATPase, to (b) 0.1 mM trifluoperazine, an agent which renders calmodulin ineffective, and to (c) 10 \( \mu \)M neomycin sulfate, which blocks release of Ca^{2+} from intracellular stores. Thapsigargin (5 \( \mu \)M), an inhibitor of Ca^{2+}-ATPase of the endoplasmic reticulum, prolonged fluorescence. These observations indicate that cell surface fluorescence was due to the capture of Ca^{2+} by Calcium Green C_{18} after Ca^{2+} had been translocated across osteoblast plasma membranes. Involvement of the plasma membrane Ca^{2+}-ATPase, known to be present in osteoblasts in substantial amounts, is implicated.

Osteoblasts line bone surfaces at sites of bone formation and secrete a protein matrix that has the capacity to calcify. The precise manner by which Ca^{2+} passes through the layer of osteoblasts to sites of calcification is not known. The demonstration of substantial amounts of a plasma membrane Ca^{2+}-ATPase in osteoblasts (Akiyama et al., 1988) raised the interesting possibility that the enzyme may be involved in movement of Ca^{2+} to calcifying sites in bone. More recently, a plasma membrane Ca^{2+}-ATPase has been cloned and sequenced from human osteoblast cell lines (SAOS-2; HOBIT) and shown to bear high sequence homology to other plasma membrane Ca^{2+}-ATPases (Kumar et al., 1993). In a preparation of vesicles derived from osteoblast plasma membranes, the magnitude and direction of calcium translocation has been determined (Gay and Lloyd, 1995). Sealed inside-out vesicles took up Ca^{2+}, implying that the direction of pumping in cells was outward, as has been found for numerous other cell types (Carafoli, 1987; Garrahan and Rega, 1990). The uptake rate by osteoblast plasma membrane vesicles was 9.9 ± 2.3 nmol/mg of protein/min, which falls in the range reported for several other vesicle studies, including red blood cells (Caride et al., 1983), parotid gland (Takuma et al., 1985), neutrophils (Ochs and Reed, 1983), and liver (Kraus-Friedmann et al., 1982). Na^{+}/Ca^{2+} exchange also exists in osteoblasts (Krieger, 1992; Short et al., 1994) and may contribute in a minor way to the Ca^{2+} efflux observed, as subsequently discussed.

The present study was undertaken to establish characteristics of the fluorophore, Calcium Green C_{18}, when applied to living cells and to utilize the dye to evaluate calcium efflux in intact osteoblasts. Parathyroid hormone was employed to stimulate an increase in [Ca^{2+}]_{i}. This occurs as a consequence of Ca^{2+} release from intracellular stores as has been shown in other studies of osteoblasts (e.g. Reid et al., 1987; Civitelli et al., 1989). It was anticipated that the return of [Ca^{2+}]_{i} to pre-stimulatory levels would involve, in part, efflux by plasma membrane Ca^{2+}-ATPase activity, since this enzyme system is a major pathway utilized by cells to restore [Ca^{2+}]_{i} to basal levels (Carafoli et al., 1990). The lipophilic calcium-sensitive fluorescent dye, Calcium Green C_{18}, was selected as a likely candidate for monitoring Ca^{2+} efflux. Since the fluorophore is hydrophilic and is negatively charged, it is excluded from the lipid bilayer; the lipophilic hydrocarbon chain lodges in the plasma membrane, holding the dye in close proximity to the membrane. We reasoned that Ca^{2+}, upon emerging from the cell, would be trapped by the fluorophore, which would then fluoresce. A laser scanning confocal imaging system was used to visualize sites of calcium efflux and to measure both the intensity and duration

---

*This work was supported by National Institutes of Health Grant DE09459. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: 108 Althouse Laboratory, University Park, PA 16802. Tel.: 814-865-6722; Fax: 814-863-7024.

1The abbreviations used are: [Ca^{2+}]_{i}, cytosolic free Ca^{2+} concentration; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid; DMF, N,N-dimethylformamide; DOPC, 1,2-dioleoylphosphatidylcholine; MOPS, 3-(N-morpholino)propanesulfonic acid; PTH, parathyroid hormone (bovine, 1-84); RCBS5, reduced-calcium balanced salt solution; IP_{3}, inositol 1,4,5-trisphosphate.
Calcium Green-1. The synthesis starts with catalytic reduction of 5,5'-pure Calcium Green C18 tetrapotassium salt. The synthesis of Calcium fluorescent product is purified by reverse-phase chromatography to give and dichlorofluorescein are hydrolyzed with base. The resulting soapy protected dichlorofluorescein, and the protecting group on the BAPTA is shown in Fig. 1.

Under vacuum to a gray oil, which is stirred with 200 ml of methanol for 30 min; 3.6 ppm of 12H, s; 4.1 ppm of 4H, s; 4.15 ppm of 4H, s; 4.2–4.4 ppm of 4H, d; 6.8 ppm of 2H; 7.05 ppm of 2H; 7.3 ppm of 2H, s.

Synthesis of Compound II: N-Stearoyl-5,5'-bis- amino-BAPTA Tetramethyl Ester—A solution of stearoyl chloride (1.6 g; 5.4 mmol) in 20 ml of dry DMF and shaken under hydrogen at 40 p.s.i. for 3 h in the presence of 0.8 g of 10% palladium on charcoal. TLC in ethyl acetate shows the formation of a new single product that is colorless and gives a brown/red product on reaction with ninhydrin. The catalyst is removed by filtration through a diastomaceous earth, and the colorless filtrate is concentrated under vacuum to a gum, which is dissolved in 200 ml of CH2Cl2 and 1 ml of dry methanol, then stirred with 0.189 g of Compound III in 1 ml of dry methanol for 30 min.

DEFINING CONDITIONS FOR LOADING CALCIUM GREEN C18 INTO OSTEOBLASTS—Primary osteoblasts were isolated as described previously (Gay et al., 1994). Briefly, cells were scraped from periosteal surfaces of 3–week-old chick tibias, after mild enzyme treatment, and cultured on 25-mm glass coverslips in Dulbecco’s modified Eagle’s medium (Sigma) plus 10% heat-inactivated fetal bovine serum for 6 to 8 days.

Introduction of Calcium Green C18 into the plasma membranes in vivo is essential for obtaining information about the dynamics of calcium in living cells. Calcium Green C18 was tested for response to free Ca2+ when excited at 488 nm. Calcium Green C18 was tested for response to free Ca2+ both in aqueous solution and in the presence of DOPC liposomes at a dye concentration of 1 μM. To create liposomes, 100 μl of 40 mM DOPC (in ethanol) was injected slowly (over 1 min) into a buffer, containing 25 mM HEPES buffer at pH 7.3, ionic strength 0.1 M, 37°C, 10% fetal bovine serum plus 0.1 mM Ca2+.

Propidium iodide (2 μg/ml) was present in the RCBSS in some experiments as a test of cell viability.

DEFINING CONDITIONS FOR LOADING CALCIUM GREEN C18 INTO OSTEOBLASTS—Primary osteoblasts were isolated as described previously (Gay et al., 1994). Briefly, cells were scraped from periosteal surfaces of 3–week-old chick tibias, after mild enzyme treatment, and cultured on 25-mm glass coverslips in Dulbecco’s modified Eagle’s medium (Sigma) plus 10% heat-inactivated fetal bovine serum for 6 to 8 days.

Embryonic osteoblasts were isolated from distal tibiae of 16-day chick embryos by a modification of the method of Brinckerhoff and Wakil (1984). The cells were then exposed to 1, 2, 5, or 10 μM with a final concentration of 0.1 M Ca2+.

Synthesis of Compound IV: Calcium Green C18, Hexapotassium Salt—Calcium Green-1 is a fluorescent indicator of free Ca2+ ions. It consists of a dichlorofluorescein conjugated to an aromatic Ca2+ binding site (BAPTA) through an amide spacer (Haugland, 1992). This indicator can be excited at 488 nm and gives an increase in emission intensity at 530 nm on reversibly binding Ca2+, with a Kd for Ca2+ of 0.189 μM. The increase in emission intensity for free Calcium Green-1 in solution is approximately 12-fold. Dichlorofluorescein was chosen as the fluorophore for the synthesis of Calcium Green because it is less pH-sensitive than fluorescein itself and still retains a high quantum yield (0.75 at saturating Ca2+ ) when excited at 488 nm.

Based on previous experience with the synthesis of a lipophic Fura-2 (Etter et al., 1994), we synthesized a lipophic derivative of Calcium Green-1. The synthesis starts with catalytic reduction of 5,5'-dinitro-BAPTA tetramethyl ester to the diamine (Pethig et al., 1989), which is then reacted with 1 eq of stearyl chloride to give the mono-substituted lipophic BAPTA tetramethyl ester. This is conjugated to a protected dichlorofluorescein, and the protecting groups on the BAPTA and dichlorofluorescein are hydrolyzed with base. The resulting soapy protected dichlorofluorescein, purified by reverse-phase chromatography, is shown in Fig. 1.

Defining Conditions for Loading Calcium Green C18 into Osteoblasts—Primary osteoblasts were isolated as described previously (Gay et al., 1994). Briefly, cells were scraped from periosteal surfaces of 3–week-old chick tibias, after mild enzyme treatment, and cultured on 25-mm glass coverslips in Dulbecco’s modified Eagle’s medium (Sigma) plus 10% heat-inactivated fetal bovine serum for 6 to 8 days.

Introduction of Calcium Green C18 into the plasma membranes in vivo is essential for obtaining information about the dynamics of calcium in living cells. Calcium Green C18 was tested for response to free Ca2+ when excited at 488 nm. Calcium Green C18 was tested for response to free Ca2+ both in aqueous solution and in the presence of DOPC liposomes at a dye concentration of 1 μM. To create liposomes, 100 μl of 40 mM DOPC (in ethanol) was injected slowly (over 1 min) into a buffer, containing 25 mM HEPES buffer at pH 7.3, ionic strength 0.1 M, 37°C, 10% fetal bovine serum plus 0.1 mM Ca2+.

Propidium iodide (2 μg/ml) was present in the RCBSS in some experiments as a test of cell viability.

DEFINING CONDITIONS FOR LOADING CALCIUM GREEN C18 INTO OSTEOBLASTS—Primary osteoblasts were isolated as described previously (Gay et al., 1994). Briefly, cells were scraped from periosteal surfaces of 3–week-old chick tibias, after mild enzyme treatment, and cultured on 25-mm glass coverslips in Dulbecco’s modified Eagle’s medium (Sigma) plus 10% heat-inactivated fetal bovine serum for 6 to 8 days.

Introduction of Calcium Green C18 into the plasma membranes in vivo is essential for obtaining information about the dynamics of calcium in living cells. Calcium Green C18 was tested for response to free Ca2+ when excited at 488 nm. Calcium Green C18 was tested for response to free Ca2+ both in aqueous solution and in the presence of DOPC liposomes at a dye concentration of 1 μM. To create liposomes, 100 μl of 40 mM DOPC (in ethanol) was injected slowly (over 1 min) into a buffer, containing 25 mM HEPES buffer at pH 7.3, ionic strength 0.1 M, 37°C, 10% fetal bovine serum plus 0.1 mM Ca2+. The cells were then exposed to 1, 2, 5, or 10 μM with a final concentration of 0.1 M Ca2+.
exposed to 1.2 mM CaCl₂ added to RCBSS which was added through a port in the controlled environment chamber. Patterns of fluorescence were observed in optical sections using the 488 nm laser line, zoom 1.0, 10% neutral density filter, aperture opening 2.7 mm, gain 8, and black level 2 settings on the confocal microscope (Bio-Rad MRC 600). For a control, cells were treated with 50 mM EGTA for 5 min in order to chelate added calcium.

In order to assess the extent of photobleaching, cells were loaded with 5 μM Calcium Green C₁₈ for 10 min, then placed in buffer that contained 1.2 mM CaCl₂, and exposed to the laser beam every 5 s for 5 min (Table I).

Characterization of Ca²⁺ Efflux in Osteoblasts Using Calcium Green C₁₈—As an Ion Trap—In order to characterize Ca²⁺ efflux by osteoblasts, cells loaded with Calcium Green C₁₈ were placed in the Dvorak-Stotler chamber in RCBSS, and a bolus (20 μl) of PTH (final concentration 10⁻⁸ M) was injected through a port into the chamber as a means of increasing [Ca²⁺]i. Optical sections, 1.0 micron in thickness, in the x-y plane through cell centers were recorded every 5 s for the first 200 s, then every 60 s thereafter, depending on the duration of the fluorescence due to Ca²⁺ efflux. Under the chosen settings for the confocal system, the background fluorescence caused by the trace amount of Ca²⁺ present in RCBSS was negligible. Controls included: thapsigargin (5 μM), an inhibitor of endoplasmic reticulum Ca²⁺-ATPase; trifluoperazine (0.1 μM), an activator of calmodulin; quercetin (10 μM) and sodium vanadate (10 μM), inhibitors of plasma membrane Ca²⁺-ATPase; and neomycin sulfate (10 μM), an inhibitor of phospholipase C and therefore of generation of IP₃, the intracellular signal that causes Ca²⁺ release from intracellular stores. Cells were pre-incubated with the inhibitors in RCBSS for 10 min at room temperature. RCBSS in the culture chamber also contained the same concentration of each inhibitor. Fluorescence images were recorded, and image analysis was carried out using COMOS commands in the Bio-Rad software package. The image space was divided into pixels (0.17 μm² when using the 40X objective lens) and assigned a gray level (on a scale of 1-256) as a measure of fluorescence intensity.

Quercetin, sodium orthovanadate, neomycin sulfate, trifluoperazine, thapsigargin, and HEPES buffer were obtained from Sigma. PTH (bovine, 1-84) was obtained from the National Hormone and Pituitary Agency (Bethesda, MD).

RESULTS

Synthesis and Fluorescence Excitation Spectra of Calcium Green C₁₈—The structures of the compounds synthesized, then utilized in the synthesis of Calcium Green C₁₈, described under “Experimental Procedures,” are diagramed in Fig. 1. Fig. 2 shows the excitation spectra of 1 μM Calcium Green C₁₈ in the presence of increasing concentrations of Ca²⁺ in aqueous solution. As [Ca²⁺] increased, the emission intensity (at 530 nm) increased with little shift in wavelength.

Localization and Characterization of the Lipophilic Calcium Green C₁₈ in Cells—Cells loaded with Calcium Green C₁₈ that were exposed to 1.2 mM CaCl₂ appeared as rings in optical sections produced by confocal microscopy (Fig. 3A). A low level of cytoplasmic staining was also evident in many cells. This was, however, a minor contribution to total cell fluorescence. Occasionally, cell cytoplasm was intensely fluorescent; this phenomenon coincided with cell death, as these cells were found to stain with propidium iodide. Following the addition of EGTA to the observation chamber, fluorescence of cell peripheries were markedly reduced (Fig. 3B; Table II). Controls indicated that photobleaching contributed only to a minor extent to the disappearance of the fluorescent images; as shown in Table 1, loss of fluorescence due to photobleaching after a total of 60 scans over a time frame of 5 min ranged between 3 and 13%. Serial optical sections along the z axis of dye-loaded cells further revealed that Calcium Green C₁₈ was evenly located along peripheries of cells (Fig. 4).

Characterization of Calcium Efflux—The plasma membrane of approximately 30% of the 8-day-old cultured osteoblasts were found to fluoresce following PTH stimulation. A rapid and transient increase in fluorescence was observed on the surface of the responding osteoblasts when PTH was added to the cell chamber (Fig. 5). The initial efflux was usually detectable 10 s after the addition of PTH. Fluorescence intensity peaked around 2-3 min and persisted for about 7-10 min. Cell shape changes, including the appearance of cell processes, were observed in some cases after PTH stimulation (Fig. 5). Quercetin, sodium vanadate, trifluoperazine, and neomycin sulfate substantially impaired Ca²⁺ efflux, both in magnitude and duration (Table III). Thapsigargin caused the fluorescence to persist for 15-17 min (Table III), nearly twice the time found for untreated controls.

DISCUSSION

In this paper we have described the synthesis and spectral properties of a lipophilic long-chain Ca²⁺-sensitive fluorescent dye, Calcium Green C₁₈, that localizes preferentially in the plasma membrane of cells. Our goal was to utilize this fluorescent Ca²⁺-specific probe to monitor Ca²⁺ translocation out of osteoblasts. The Ca²⁺-trapping head group of Calcium Green C₁₈ facing the external medium served as a reporter of Ca²⁺ efflux. Conjugation of Calcium Green-1 with a lipophilic tail, 18 carbons long, as shown in Fig. 1, provided a means of situating the fluorescent probe along a lipid-based membrane while preserving the capacity of the indicator to fluoresce. It is likely that the method of loading the dye achieved placement of most of the dye into the plasma membrane with the fluorophore facing the outward direction since added external EGTA abolished fluorescence. Furthermore, the observation that the plasma membrane Ca²⁺-ATPase inhibitors, quercetin, vanadate and trifluoperazine, greatly reduced membrane fluorescence (Table III) indicates that most of the Calcium Green C₁₈ was loaded in the preferred orientation. If substantial amounts of Calcium Green C₁₈ had been inwardly oriented, the dye would have fluoresced when [Ca²⁺] increased whether Ca²⁺ efflux was inhibited or not. While some fluorescence was observed in the cytosol, it was minor compared to the cell periphery under short (10 min) loading times. At the longer loading times, internal fluorescence was common. Using three-dimensional imaging techniques Calcium Green C₁₈ has been found useful for demonstrating Ca²⁺ efflux from primary osteoblasts stimulated with parathyroid hormone. It was also possible to monitor the duration of the response. This is the first study to demonstrate that Calcium Green C₁₈ can be used to detect Ca²⁺ efflux from cells.

The spectral response of Calcium Green C₁₈ to increasing concentrations of Ca²⁺ in aqueous solution, where the Kₘ was 0.23 μM, shows a linear increase in emission intensity in the submicromolar range (Fig. 2). In this state, the lipid tail of the dye is not embedded in a membrane, so the response is expected to differ slightly from that in cells. In the presence of DOPE liposomes (spectra not shown), a linear increase in fluorescence with increasing concentrations of Ca²⁺ was also observed, but the affinity of the dye for Ca²⁺ was higher (Kₘ = 0.062 μM). The water solubility of this probe is better than for C₂₀Fura-2, as Calcium Green-1 has an extra negative charge. This should facilitate loading the dye into cell membranes from

---

**Table I**

| Field of view | Photon counts/field | Number of cells/field | % change (± S.E.) |
|---------------|---------------------|-----------------------|------------------|
|               | Scan 1 | Scan 60              |                   |
| 1             | 22.03  | 19.29                | -12              |
| 2             | 7.90   | 7.28                 | -8               |
| 3             | 9.56   | 9.07                 | -5               |
| 4             | 30.65  | 26.79                | -13              |
| 5             | 13.98  | 13.54                | -3               |

\[ \Delta = -8.2 \pm 0.8 \]
an aqueous solution. Further, once in place in the membrane, dye-dye interactions would be reduced due to the negativity of the head group.

In cultured osteoblasts, Calcium Green C18 appears to be localized along the entire plasma membrane, except for regions associated with the coverslip. Calcium specificity is indicated since the calcium greens have been shown to preferentially bind calcium (Girard et al., 1992). Further, little fluorescence was observed under the chosen confocal settings when cells were bathed with RCBSS, which intrinsically contained trace amounts of Ca$^{2+}$. Fluorescence was induced when Ca$^{2+}$ was added to the RCBSS (Fig. 3A) and then largely disappeared when the Ca$^{2+}$-specific chelating agent EGTA was added externally (Fig. 3B). Because of the special orientation of Calcium Green C18 and its relatively low $K_d$, it is critical that trace amounts of calcium present in the RCBSS be small. At a Ca$^{2+}$ concentration present in the RCBSS of 0.22 $\mu$M, $50\%$ of the indicator would be free to bind calcium emerging from the cell. The $K_d$ measured in aqueous solution, rather than in liposomes, is considered to better represent the $K_d$ of the dye intercalated into the plasma membrane since the fluorescent head group is entirely extracellular and is bathed in aqueous solution.

Another near-membrane Ca$^{2+}$ concentration indicator has been described recently that consists of Fura-2 conjugated to a lipophilic C18 alkyl chain (Etter et al., 1994). C18-Fura-2 orients in the plasma membrane so that the fluorophore faces from the side to which it was applied. This indicator was found to detect rapid, localized changes in [Ca$^{2+}$]i which are undetectable by water-soluble bulk cytosolic fluorescent Ca$^{2+}$ indicators. Calcium Green C18, on the other hand, has a higher negative charge, which would assist in maintaining the outward orientation of the dye in the plasma membrane due to the negative membrane potential of intact cells. In addition, the negativity of Calcium Green C18 would result in fewer dye-dye interactions once the dye is positioned in the cell membrane. The calcium greens are useful for kinetic analysis due to increased quantum yields on binding Ca$^{2+}$ (Haugland, 1993). However, since the calcium greens are not ratiometric dyes, they are not useful for measuring ion concentrations accurately. The lower $K_d$ of Calcium Green C18 in liposomes than for C18-Fura-2 (0.062 $\mu$M versus 0.15 $\mu$M) indicates that Calcium Green C18 can be used to detect lower levels of Ca$^{2+}$ and therefore is more...
sensitive. In this study we did not use an EGTA/CaEGTA
buffer in the assay medium surrounding the cells because of
concerns with cell membrane instability. Use of such a buffer-
ing system, needs to be explored, however, for detection of very
low levels of Ca$^{2+}$. The improved sensitivity of the calcium
greens has been discussed by Eberhard and Erne (1991). In
addition, the calcium greens show increased specificity, speed
of response, and higher spatial and temporal resolution
(Haugland, 1992). Advantages of Calcium Green C$_{18}$ being
excitable at longer wavelengths over that of C$_{18}$-Fura-2 include
less cellular photodamage and decreased autofluorescence.
In addition, Fura-2 may bind to cellular proteins and could lead
to alterations in the response of the indicator to Ca$^{2+}$ (Blatter
and Wier, 1990).

The present study is the first direct demonstration of Ca$^{2+}$
efflux from osteoblasts. The source of Ca$^{2+}$ is believed to be
from intracellular stores since cytosolic calcium in osteoblast
cell lines has been shown to increase following PTH treatment
Peripheral fluorescence of cells prior to and following EGTA treatment

| Field of view | Photon counts/field | Number of cells/field | % change (± S.E.) |
|---------------|---------------------|-----------------------|------------------|
|               | Before              | After                 |                  |
| 1             | 11.37               | 1.33                  | 3                |
| 2             | 13.90               | 2.04                  | 8                |
| 3             | 20.04               | 1.01                  | 8                |
| 4             | 23.19               | 3.29                  | 8                |
| 5             | 17.40               | 2.99                  | 8                |
|                |                     |                       | \( -48.74 \pm 1.73 \) |
Ca\textsuperscript{2+} Efflux of Osteoblasts

Philipson, K., and Nicoll, D. (1993) Int. Rev. Cytol. 137, 199–227
Redl, I. A., Civitelli, R., Halstead, L. R., Avidi, L. V., and Hruska, K. A. (1987) Am. J. Physiol. 253, E45-E51

Ribeiro, C. P., and Mandel, L. J. (1992) Am. J. Physiol. 262, F209-F216
Sasaki, T., and Garant, P. R. (1987) Cell Tissue Res. 248, 103-110
Short, C. L., Monr, R. D., Bushinsky, D. A., and Krieger, N. S. (1994) J. Bone Miner. Res. 9, 1159-1166
Takuma, T., Kuyatt, B. L., and Baum, B. J. (1985) Biochem. J. 227, 239–245
Thastrup, O., Foder, B., and Scharff, D. (1987) Biochem. Biophys. Res. Commun. 142, 654–660

Tsien, R., and Pozzan, T. (1989) Methods Enzymol. 172, 230–262
van Leeuwen, J. P. T. M., Bos, M. P., Lowik, C. W. G. M., and Herrmann-Erlee, M. P. M. (1986) Bone Miner. 4, 177-188
Wüthrich, A., and Schatzmann, H. J. (1980) Cell Calcium 1, 21–33
Yamaguchi, D. T., Green J., Kleeman C. R., and Muallem S. (1991) Cell Calcium 12, 609-622