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A Calcium Release Activated Calcium Influx in Primary Cultures of Rat Osteoblast-like Cells

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Abstract. Osteoblast-like (OBL) cells in primary culture were tested for their ability to generate a calcium release activated calcium flux (CRAC). Influx of Ca^{2+} was optically detected by fura-2. Intracellular calcium stores (ICS) were emptied in the absence of extracellular calcium ($[Ca^{2+}]_e$) by 5 μM thapsigargin (TG) or 2 μM A23187. Readdition of 1.8 mM $[Ca^{2+}]_e$ increased the free intracellular Ca^{2+} ($[Ca^{2+}]_i$) after a delay of 30–60 seconds at a rate of 2.3 nM/s due to CRAC. This rate depended on $[Ca^{2+}]_e$ and was substantially lowered if readdition of 1.8 mM $[Ca^{2+}]_e$ was preceded by, e.g., 0.72 mM $[Ca^{2+}]_e$. CRAC-induced $[Ca^{2+}]_i$ peaks were correlated ($r = 0.543$) with $[Ca^{2+}]_i$ peaks during the complete depletion of ICS with A23187. Ca^{2+} influx due to CRAC could be blocked by flufenamic acid (100 μM) but not verapamil (20 μM). Ni^{2+} (1 mM), although reversibly blocking CRAC, accelerated the initial $[Ca^{2+}]_i$ influx rate. Induction of CRAC enhanced the influx of Mn^{2+} 4.3-fold, as measured by quenching of fura-2 fluorescence. In summary, OBL cells exhibit a CRAC which allows for the permeation of ions other than Ca^{2+}. This Ca^{2+} flux may be activated by transmembraneous gradients of Ca^{2+} and Ni^{2+}.

Key words: Capacitative calcium current — Thapsigargin — Ni^{2+} — Flufenamic acid — Mn^{2+}.

The main task of osteoblasts in bone is the formation of extracellular osteoid which is successively mineralized by the deposition of phosphorus and calcium (Ca^{2+}). This process leads to a ratio of Ca^{2+} and phosphorus which is approximately 2 in bone and also in mineralizing cultures of osteoblast-like (OBL) cells [1]. It has been described that *in vitro* OBL cells secrete Ca^{2+} after an elevation of the free intracellular calcium concentration ($[Ca^{2+}]_i$) due to depletion of intracellular calcium stores (ICS) after, e.g., a parathyroid hormone stimulus [2, 3]. Thus, it appears that Ca^{2+} stemming from ICS may be crucial not only for intra- and intercellular signaling [4–6] but also contributes to mineralization processes in bone, as suggested by Zimmermann et al. [7].

In nonexcitable cells, refilling of ICS often depends on a voltage-independent ''calcium release activated current'' via the cell membrane [8]. This current is activated by a hitherto unknown signal possibly released from empty ICS. Since the influx of Ca^{2+} depends on the filling state of ICS (their ''storage capacity'') it is now commonly referred to as ''capacitative calcium current'' [9]. Recent studies showed that the mammalian genes of channels responsible for this current share some homology with so-called TRP genes of Drosophila [10, 11], suggesting a widespread distribution of the gene family. Although a calcium-release activated calcium flux (CRAC) could be visualized by means of Ca^{2+}-sensitive dyes in a variety of nonexcitable cells (for overview, see [12]), an electrophysiological characterization often has been impaired by a low single channel conductance [11, 12]. This circumstance led to the assumption of alternate influx pathways of Ca^{2+} involving, e.g., membrane tubules or elements of the cytoskeleton [12, 13].

In contrast, excitable cells are thought to refill ICS via highly abundant voltage-dependent calcium currents [12, 14], although a few excitable cell types such as PC12, GH3 [14, 15], and neurons of the developing retina have been demonstrated to express a CRAC [16]. In this context, OBL cells are of special interest. On the one hand, they are assumed to be nonexcitable cells, while on the other hand, recent studies have shown that they have voltage-gated channels for Ca^{2+} and Na^{+} and are able to generate action potentials [5, 17–19]. These unique properties of OBL cells raise the question of whether they exhibit a CRAC for refilling ICS, which could be important for mineralization. To answer this question the present experiments were carried out on fura-2-stained and ICS-depleted OBL cells using a protocol of Ca^{2+} withdrawal/Ca^{2+} readdition.

Materials and Methods

Cell Cultures

Calvarial fragments derived from newborn rats were explanted onto collagen-coated coverslips (24 × 36 mm, cf [6]). Rat OBL cells were maintained at 35°C in HEPES (20 mM) buffered minimum essential medium (MEM) supplemented with glutamine (2 mM), penicillin (50 IU/ml), streptomycin (50 μg/ml), and 10% fetal calf serum (FCS). After 1 week, FCS was reduced to 5%. All cell culture components were from Flow Laboratories (Meckenheim, Germany). Medium was changed twice a week. Cultures were tested 8–12 weeks after explantation when cells growing directly on the coverslip were close to confluence.

Solutions and Reagents

Control saline (Hanks balanced salt solution, HBS) contained (in

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mM) NaCl 140, KCl 3, $CaCl_2$ 1.8, $MgSO_4$ 1.3, KH_2PO_4 1.25, glucose 11, and HEPES 10, pH 7.4. In some test solutions $CaCl_2$ was lowered to (in mM) 0.18 or 0.72. In EGTA-HBS the concentration of free Ca^{2+} in the saline was buffered to 100 nM using 1.4 mM $CaCl_2$ and 3 mM EGTA. Stock solutions of the nonfluorescent calcium ionophore 4-bromo-A23187 (A23187, 2–10 mM, Sigma) and 5 mM thapsigargin (TG) (Molecular Probes) were prepared in dimethylsulfoxide (DMSO); final concentration of DMSO in either type of HBS was 0.1%. Flufenamic acid, verapamil (both from Sigma) and $NiCl_2$ (Merck, analytical grade) were dissolved in HBS. Fura-2 AM and SBFI AM (Molecular Probes) were dissolved in DMSO.

Fluorescence Optical Measurements of $[Ca^{2+}]_i$

OBL cells were loaded with 5 μM fura-2 AM added to the culture medium for 1 hour at 35°C. Excessive dye was washed away three times with HBS. Thereafter, the coverslip was transferred to a perspex frame where it was used as the bottom of the experimental chamber (24.5 × 36.5 × 7 mm) which was mounted on the stage of an inverted microscope (Zeiss Axiovert 135TV, Germany). Solutions were changed within the chamber by replacing HBS with the desired fluid (at least three times in intervals of 10–15 seconds). Experiments were performed at room temperature (20 ± 2°C). Intracellular-free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the ratio method with a dual wavelength excitation (Attofluor RatioVision System, Atto Instruments, Rockville, USA). Relative changes of $[Ca^{2+}]_i$ were determined by the emission ratios (excitation 340 nm/380 nm; emission 500–530 nm), which were collected at 0.05–1 Hz and were corrected for background fluorescence. A 20× Fluar objective (Zeiss, Oberkochen, Germany) was used. Absolute $[Ca^{2+}]_i$, as given in the text was estimated according to the equation of Grynkiewicz [20]: $[Ca^{2+}] = K_d * \beta * \{(R - R_{min})/(R_{max} - R)\}$ where K_d is the dissociation constant of fura-2 binding to intracellular Ca^{2+} (assumed to be 225 nM), β is the maximal fluorescence at 380 nm divided by the minimal fluorescence at 380 nm, and R is the ratio of the two fluorescence intensities 340nm/380nm. Addition of 3 M KCl was used to obtain R_{max} values; 11 mM of the Ca^{2+} chelator EGTA was used to obtain R_{min} values in OBL cells pretreated with A23187 (5 μM).

Statistical Analysis

At least three experiments were carried out for each treatment, and in each experiment 10–50 cells were observed simultaneously. Ca^{2+} transients shown as ratio values in the figures represent averages from all cells of one representative experiment. Absolute values of $[Ca^{2+}]_i$ as given in the text represent mean ± standard deviation of the total number of cells (n values) of all experiments. For nonparametric statistical analysis, the Mann-Whitney U test was used; $\alpha \leq 0.5$ was considered significant.

Results

The aim of the present study was to follow and partially characterize the CRAC of rat OBL cells. For this purpose the free intracellular calcium concentration ($[Ca^{2+}]_i$) was measured in fura-2 AM-loaded cells. Provided that the ICS are empty, only an influx of calcium could increase $[Ca^{2+}]_i$. Keeping in mind that this increase in $[Ca^{2+}]_i$ underestimates the respective influx due to $[Ca^{2+}]_i$ buffering and Ca^{2+} extrusion by Ca^{2+}-ATPases and/or Na^+/Ca^{2+} exchange, $[Ca^{2+}]_i$ increases can be taken as a measure for the transmembraneous flux. In the present experiments, ICS were depleted by the ionophore 4-bromo-A23187 (A23187) or TG while cells were kept in a low calcium salt solution (EGTA-HBS) until $[Ca^{2+}]_i$ had declined to low and constant values. Thereafter, a transmembraneous Ca^{2+} gradient was generated by readdition of $[Ca^{2+}]_e$ allowing CRAC to occur.

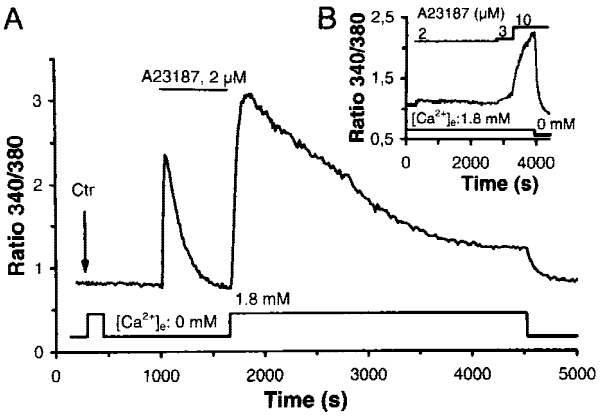

Fig. 1. Calcium release activated Ca^{2+} current (CRAC) in fura-2 loaded OBL cells elicited with 4-bromo-A23187 (A23187). **(A)** control conditions (Ctr): a transient change of $[Ca^{2+}]_e$ from 100 nM (EGTA-HBS, $[Ca^{2+}]_e$: 0 mM) to 1.8 mM failed to increase $[Ca^{2+}]_i$ (arrow). A23187 (2 μM) released Ca^{2+} from intracellular calcium stores. 1.8 mM $[Ca^{2+}]_e$ then evoked a triphasic increase of $[Ca^{2+}]_i$ characterized by a maximum (initiation phase), a long-lasting decay (decay phase), and a sustained elevation (sustained phase, average from 24 cells). **(B)** OBL cells with empty ICS (pretreated for 11 hours with 5 μM thapsigargin in culture medium and normal $[Ca^{2+}]_e$) were used to test for the contribution of A23187 to the elevation of $[Ca^{2+}]_i$ during CRAC. Ascending concentrations of A23187 evoked small transient (2 μM) or progressive (3, 10 μM) increases of $[Ca^{2+}]_i$. Initial $[Ca^{2+}]_i$ peaks were never observed (average from 24 cells).

Induction of CRAC with A23187

Besides TG, which inhibits the smooth endoplasmic reticulum Ca^{2+}-ATPase, ionophores such as ionomycin or A23187 can be used to induce CRAC [21, 22]. In this study application of 2 μM A23187 in EGTA-HBS led to a single peak $[Ca^{2+}]_i$ of 984 ± 370 nM (n = 531 cells) which returned to baseline within 5.5 ± 2.1 minutes (Fig. 1A). Thus, with this concentration of A23187 Ca^{2+} can be released from ICS of OBL cells. Thereafter, increasing the concentration of A23187 to even 10 μM did not further elevate $[Ca^{2+}]_i$ (three experiments), indicating that A23187-sensitive stores were completely emptied. Readdition of 1.8 mM Ca^{2+} to OBL cells pretreated with 2 μM A23187 revealed a large increase in $[Ca^{2+}]_i$ at a rate of 2.9 ± 1.8 nM/s, resulting in a peak value of 695 ± 465 nM. This increase was absent under control conditions, i.e., before stores were depleted (Ctr, Fig. 1A). After 40 ± 6 minutes $[Ca^{2+}]_i$ had decayed to constant elevated levels (240 ± 42 nM, n = 40) which depended on $[Ca^{2+}]_e$ (Fig. 1A). This elevated level possibly reflected ionophore-mediated Ca^{2+} flux because A23187 tends to reside within membranes and cannot be readily washed out. Therefore we tested to what extent the Ca^{2+} influx after ICS depletion was superimposed by a Ca^{2+} flux carried by the ionophore. We used OBL cells which had emptied their ICS in the presence of 5 μM TG given for 11 hours under cell culture conditions, i.e., at normal $[Ca^{2+}]_e$. These cells had adopted a slightly elevated steady state $[Ca^{2+}]_i$ of 180 ± 43 nM (n = 46). As can be seen in Figure 1B, the addition of 2 μM A23187 raised

$[Ca^{2+}]_i$ only transiently and, even more important, without the prominent initial peak. Further elevation of the concentration of A23187 to 3 μM and 10 μM only evoked the expected increase of $[Ca^{2+}]_i$. However, in this case also initial $[Ca^{2+}]_i$ peaks were missing.

The results presented led us to conclude that the major portion of the initial cytosolic $[Ca^{2+}]_i$ peak seen after ionophore treatment is not caused by Ca^{2+} flux carried by A23187. Instead, it is due to depletion of ICS as in other cells [21, 22] and therefore is referred to as ''CRAC.'' At the single cell level it was found that the $[Ca^{2+}]_i$ peak caused by ICS depletion with A23187 linearly correlated (r = 0.543) with the $[Ca^{2+}]_i$ maximum during readdition of extracellular Ca^{2+} if $[Ca^{2+}]_i$ was calculated in nM (evaluation of n = 70 cells). It should be added that after the transient treatment with A23187, ICS were partly filled again such that they could be emptied a second time after an extended filling period (not shown).

Induction of CRAC with Thapsigargin

TG is the most frequently used drug to evoke a calcium release-activated current because it does not convey an additional calcium conductance as ionophore treatment does. At a concentration of 5 μM administered in EGTA-HBS, TG increased $[Ca^{2+}]_i$ from 95 ± 21 nM to 224 ± 69 nM within a few minutes (431 cells, Fig. 1). After 23 ± 4 minutes, baseline $[Ca^{2+}]_i$ was reached again. At that time ICS were completely emptied because an application of A23187 (2–10 μM) failed to increase $[Ca^{2+}]_i$ (not shown). If cells pretreated with TG were exposed to 1.8 mM $[Ca^{2+}]_e$, a pronounced increase in $[Ca^{2+}]_i$ to 307 ± 106 nM occurred (Fig. 2). Typically, $[Ca^{2+}]_i$ increased (from t_{20} to t_{80}) at a mean rate of 2.3 ± 0.9 nM/s. A delay of 30–60 seconds was seen in all experiments although the Ca^{2+} containing solution was poured into the chamber in ca. 5 seconds. The increase of $[Ca^{2+}]_i$ was triphasic: maximum values were reached within the next 1–3 minutes and persisted for about 1 minute. This ''initiation phase'' was followed by a ''decay phase'' during which $[Ca^{2+}]_i$ progressively declined to constantly elevated levels (240 ± 52 nM, n = 164, ''sustained phase''). Despite the enormous long-lasting influx of Ca^{2+} (>30 minutes tested) ICS remained widely empty as was revealed by negligible increase in $[Ca^{2+}]_i$ of 30 nM which could be evoked by addition of A23187 (Fig. 2A).

After depletion of the stores with TG, this Ca^{2+} conductivity remained (Fig. 2B). It was furthermore tested whether the $[Ca^{2+}]_i$ overshoot typical for the initiation phase could be repeated. Up to five repetitive $[Ca^{2+}]_e$ increases and decreases to base level were carried out. Fig. 2B shows the calcium transients averaged from 31 cells. At the single cell level, the first $[Ca^{2+}]_i$ increase typically showed a clear maximum, whereas the following overshoots were missing or became progressively smaller.

Maximum and slope of the TG-evoked $[Ca^{2+}]_i$ increase depended on $[Ca^{2+}]_e$ (Fig. 3). Surprisingly, 0.18 mM of Ca^{2+}, which means a transmembraneous gradient exceeding 3 orders of magnitude (E_{ca} ca. + 95 mV) increased $[Ca^{2+}]_i$ to less than 15 nM (n = 45), whereas 0.72 mM (E_{ca} ca. + 112 mV) and 1.8 mM $[Ca^{2+}]_e$ (E_{ca} ca. + 124 mV) increased $[Ca^{2+}]_i$ to 130 ± 22 nM (n = 54) and 307 ± 106 nM (n = 431), respectively (Fig. 3A). When $[Ca^{2+}]_e$ was raised stepwise from 0.72 to 1.8 mM (Fig. 3B) the increase in $[Ca^{2+}]_i$ following addition of 1.8 mM $[Ca^{2+}]_e$ was 0.3 ± 0.1 nM/s,

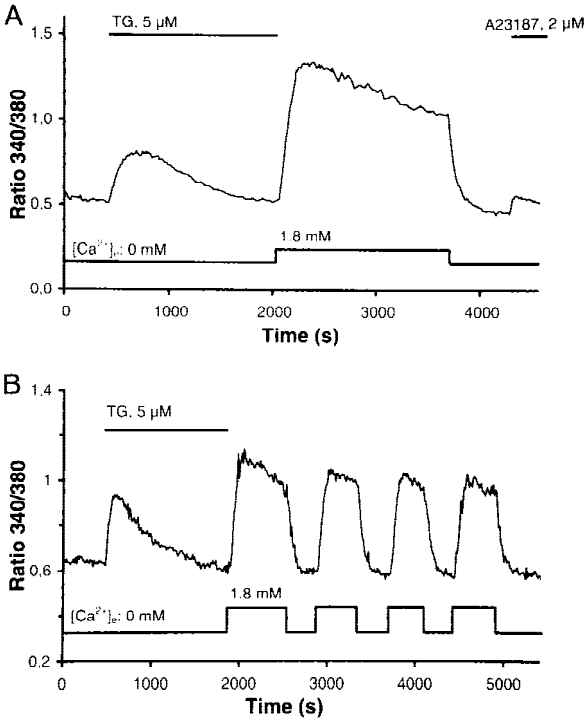

Fig. 2. CRAC in fura-2 loaded OBL cells after depletion of intracellular stores with 5 μM thapsigargin (TG) in 100 nM free extracellular Ca^{2+} (EGTA-HBS, $[Ca^{2+}]_e$: 0 mM). After $[Ca^{2+}]_i$ had declined to baseline $[Ca^{2+}]_e$ was elevated to 1.8 mM once for 30 minutes [**(A)** average from 32 cells)] or repeatedly [**(B)** average from 31 cells)]. Intracellular stores remained widely empty as is demonstrated with 2 μM A23187 in (A) Note that the initial peak values of $[Ca^{2+}]_i$ progressively decline during repeated elevation of $[Ca^{2+}]_e$ in (B).

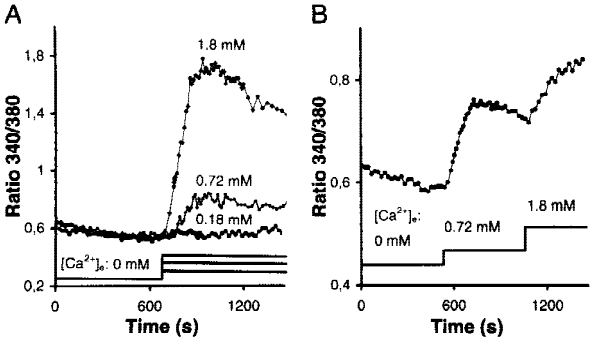

Fig. 3. Dependence of CRAC on $[Ca^{2+}]_e$ **(A, B).** Depletion of ICS with 5 μM TG (not shown); influx of Ca^{2+} was tested with different $[Ca^{2+}]_e$ levels. **(A)** $[Ca^{2+}]_e$ adjusted to (in mM) 0.18, 0.72, and 1.8 (averages from 19, 23, and 29 cells, respectively). Ca^{2+} transients of three independent experiments are superimposed. **(B)** $[Ca^{2+}]_e$ was raised from 0.72 to 1.8 mM. Increase in $[Ca^{2+}]_i$ occurred at a substantially lower rate compared with the immediate application of 1.8 mM $[Ca^{2+}]_e$.

which was about 13% the control value (2.3 nM/s, direct application of 1.8 mM Ca^{2+}, see above).

From these experiments it is concluded that the depletion of ICS with TG followed by readdition of Ca^{2+} initiates a long-lasting Ca^{2+} conductance in OBL cells, i.e., a calcium release activated Ca^{2+} flux (CRAC).

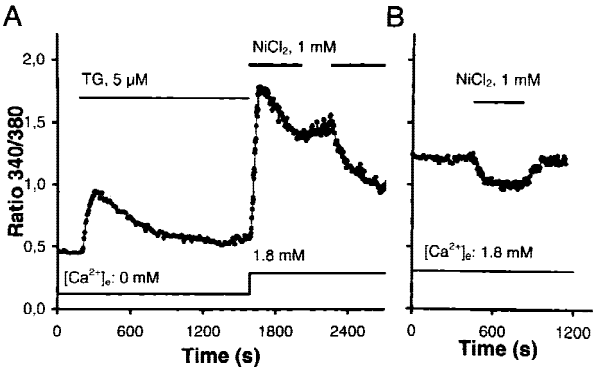

Fig. 4. Influence of Ni^{2+} on CRAC of OBL cells. Procedure as in Figure 2A. **(A)** 1.8 mM Ca^{2+} added together with 1 mM Ni^{2+} steeply increased $[Ca^{2+}]_i$ during the initiation phase. Removal of Ni^{2+} during the decay phase further increased $[Ca^{2+}]_i$. **(B)** Reversible inhibition of CRAC by Ni^{2+} during the sustained phase. Ni^{2+} was applied 1 hour after the onset of TG treatment (average of 18 cells).

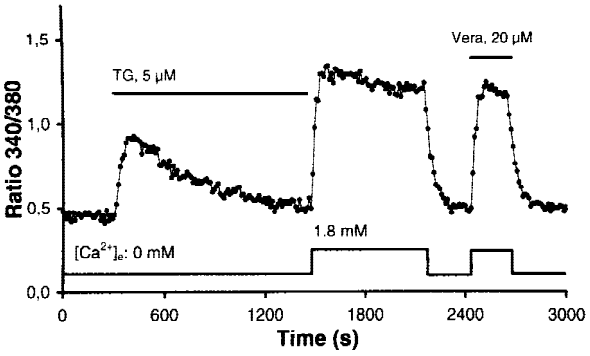

Fig. 5. Ineffectiveness of verapamil to inhibit CRAC induced by TG treatment and pulsed application of 1.8 mM $[Ca^{2+}]_i$ (average of 34 cells).

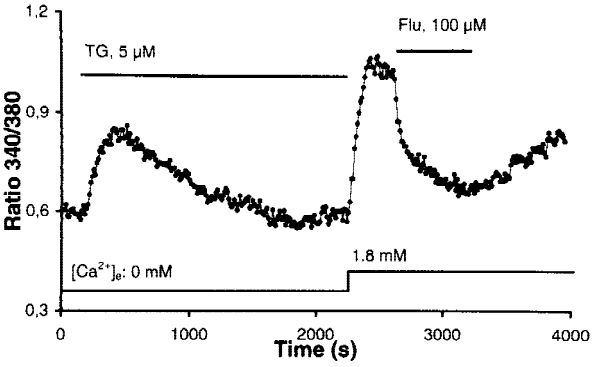

Fig. 6. Reversible inhibition of CRAC by flufenamic acid (Flu). Procedure as in Figure 2A. Flu (100 µM) was applied during the early decay phase (average of n = 17 cells).

Block of the Calcium Release Activated Ca^{2+} Flux

After ICS depletion with TG, influx of Ca^{2+} could be reversibly blocked by Ni^{2+} (1 mM, Fig. 4) which is frequently used to block channels mediating CRAC [15, 21, 23]. However, if Ni^{2+} was applied together with 1.8 mM $[Ca^{2+}]_e$ during the initiation phase (see above), the slope of $[Ca^{2+}]_i$ was 7.3 ± 1.8 nM/s (n = 44, Fig. 4A). This rate was significantly ($\alpha < 0.05$, Mann-Whitney U test) different from control cultures of the same age (4.1 ± 1.3 nM/s, n = 57) and even the minimum slope of $[Ca^{2+}]_i$ observed during application of Ni^{2+} (3.8 nM/second) exceeded the average of all other cultures tested (2.3 nM/second, n = 431). Nevertheless, the removal of Ni^{2+} during the decay phase (Fig. 4A) unblocked Ca^{2+} influx and further increased $[Ca^{2+}]_i$. This reversible blocking effect was also found during the sustained phase (Fig. 4B). Thus, it is concluded that Ni^{2+} accelerates Ca^{2+} influx during the initiation phase but clearly blocks it later on. In contrast to Ni^{2+}, the selective L-type calcium channel blocker verapamil (20 µM) did not inhibit CRAC of OBL cells when provided together with an increase in $[Ca^{2+}]_e$ (n = 91, Fig. 5). Similarly, a 10 minute lasting preapplication of 20 µM verapamil failed to inhibit CRAC (n = 17, 1 experiment). Flufenamic acid (100 µM, n = 50) which blocks nonselective channels including CRAC channels [24–29], was found to reversibly inhibit the CRAC of OBL cells, such that $[Ca^{2+}]_i$ declined with a time constant of 2–3 minutes (Fig. 6).

Influx of Manganese During CRAC

To test whether Mn^{2+} can also enter OBL cells during CRAC, as is the case in other cells [8, 30–32], Mn^{2+}-quenching experiments were performed. Mn^{2+} strongly diminishes fura-2 fluorescence. Thus, a loss of fluorescence directly indicates the permeation of the ion. Indeed, Figure 7 shows a decay of the relative emission intensity (excitation 340 nm, emission >520 nm) during application of 2 mM Mn^{2+}. Simultaneously, the ratio 340/380, i.e., $[Ca^{2+}]_i$, was not changed. Under control conditions (untreated OBL cells with filled ICS, Ctr in Fig. 7) Mn^{2+} decreased the emission intensity of fura-2 fluorescence by 4% within 30 seconds (n = 24; revealed by linear regression). After depletion of ICS with TG, dye quenching was enhanced to 43% within the same period (n = 44). Thus, the CRAC evoked increase in Mn^{2+} quenching ($\Delta E_{520}/\Delta t$) was calculated to be 4.3-fold during the sustained phase.

Discussion

Depletion of ICS of OBL cells with TG or 4-bromo-A23187 (A23187) was demonstrated to evoke a prominent CRAC flux roughly correlated with the amount of Ca^{2+} liberated from ICS, as shown for other cells [15]. The channels mediating the CRAC of OBL cells allowed not only an influx of Ca^{2+}, but also of Mn^{2+}. Influx of Ca^{2+} was inhibited by the nonselective ion channel blocker flufenamic acid but not by verapamil, whereas Ni^{2+} clearly reduced CRAC during the sustained phase although it accelerated the initiation of the current.

From the data it appears that A23187 is more potent to induce CRAC than TG and that, therefore, different types of ICS might be involved. However, $[Ca^{2+}]_i$ of TG-treated OBL cells could not further be increased with A23187 under Ca^{2+}-free conditions. This finding strongly supports the idea that A23187 affects TG-sensitive stores. The quantitative difference in CRAC induced by either A23187 or TG (peak values 695 nM versus 307 nM) could thus be due to a pronounced effectiveness in ICS depletion by A23187 and

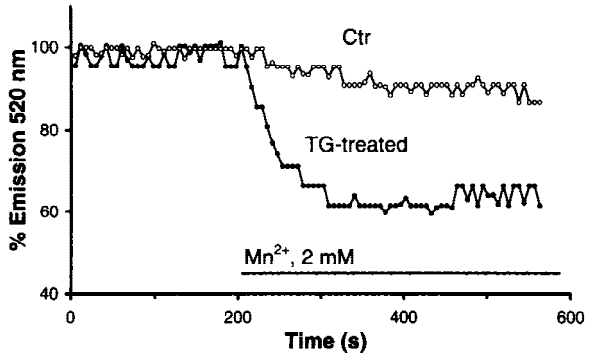

Fig. 7. Influx of manganese during CRAC. Fura-2-loaded cells kept in HBS (1.8 mM $[Ca^{2+}]_e$) were illuminated with $\lambda = 340$ nm; intensity of emitted light (>520 nm) prior to Mn^{2+} application was normalized to 100%. Control cells possessed filled intracellular stores (Ctr, average of 14 cells). CRAC was induced with 5 μM thapsigargin for 25 minutes as in Figure 2A (TG-treated, average of 23 cells). Quenching of fura-fluorescence by Mn^{2+} was enhanced indicating increased influx of Mn^{2+} during CRAC.

may involve, e.g., an increased formation of molecules which transfer the information of depleted ICS to the cell membrane.

The increase of $[Ca^{2+}]_i$ due to CRAC, as measured with fura-2 fluorescence, was triphasic: an overshoot of $[Ca^{2+}]_i$ during the initiation phase was followed by a decay to a constant plateau (sustained phase). The latter likely reflects a stable steady state balanced by Ca^{2+} influx and efflux. The constant influx persisting for hours may be explained by the irreversible blockade of the smooth endoplasmic reticulum Ca^{2+} ATPase [13] by TG such that ICS remain empty (see Fig. 1) and CRAC continues. The $[Ca^{2+}]_i$ overshoot of the initiation phase may be interpreted as a delayed onset of Ca^{2+} extrusion and/or an enhanced Ca^{2+} influx [33]. A clear distinction between these possibilities cannot be drawn on the basis of our results. However, there is some evidence that the early influx of Ca^{2+} may be facilitated, e.g., by Ca^{2+} itself: a typical delay of 30–60 seconds, which was also described for the CRAC activation of other cells [21, 32, 34], preceded the increase of $[Ca^{2+}]_i$. If ICS depletion alone had opened CRAC channels, the readdition of extracellular Ca^{2+} likely increased $[Ca^{2+}]_i$ earlier than after the delay of >30 seconds. Also, at a concentration of 0.18 mM $[Ca^{2+}]_e$, which means an E_{ca} of ca. + 95 mV, $[Ca^{2+}]_i$ should increase [cf. 35]. Moreover, if maximum $[Ca^{2+}]_i$ increases obtained at a $[Ca^{2+}]_e$ of 0.18, 0.72, and 1.8 mM would be plotted against respective E_{ca} values, an exponential rather than a linear function was obtained, suggesting that Ca^{2+} enhances its own influx. If $[Ca^{2+}]_e$ was raised from 0.72 to 1.8 mM, the rate of rise of $[Ca^{2+}]_i$ was not 2.3 nM/second as might be expected due to the transmembraneous gradient but only 13% that value (see Fig. 3B). A similar potentiation of the Ca^{2+} influx by $[Ca^{2+}]_e$ but not $[Ca^{2+}]_i$ has been described for T-lymphocytes [34]. Thus, if such a potentiation of CRAC exists in OBL cells it likely shows a desensitization since the maximum $[Ca^{2+}]_i$ increase due to the first readdition of Ca^{2+} could not be repeated in our experiments (see Fig. 2B).

In this context it is tempting to speculate that Ni^{2+}, when provided together with extracellular Ca^{2+}, can activate CRAC channels via a similar or even the same mechanism. An activation of CRAC by Ni^{2+} has already been found in T-lymphocytes [34] where it also seems to be independent from the obvious Ca^{2+} antagonistic action of Ni^{2+} which is exerted on open channels mediating CRAC (evident in the sustained phase, see Fig. 4B). Open questions remain whether, e.g., Ni^{2+} alters the activation of the channel or inactivates Ca^{2+} ATPases. Since Ni^{2+} may be a major constituent of metal materials used for implants in bone or dental applications, one may speculate that such an effect on osteoblasts may affect cellular functions and may be of clinical relevance. Also in osteoclasts, extracellular Ni^{2+} was found to disturb Ca^{2+} homeostasis by releasing Ca^{2+} from ICS [23].

The selectivity of channels mediating the CRAC of OBL cells was not restricted to Ca^{2+} since also Mn^{2+} was found to pass through CRAC channels (Fig. 7). This is characteristic for most [8, 30, 31] but not all channels passing CRAC [36]. The 4.3-fold increase of Mn^{2+} permeation (measured as $\Delta E_{520}/\Delta t$) in the sustained phase of CRAC is in the range of smooth muscle cells [37] and 301-cells [38]. However, quantitative values for a permeation of Mn^{2+} after ICS depletion depend on cellular differentiation [37] and thus are hardly comparable. A low selectivity of the CRAC channel of OBL cells is furthermore suggested by the finding that the intracellular Na^+ increased upon addition of TG and remained constantly elevated (data not shown).

The characteristics discussed so far strongly suggest that OBL cells express a nonselective CRAC. This is also suggested by the inhibitory action of flufenamic acid (Fig. 6). This drug has antiproliferant [27] as well as antiinflammatory properties [28] and blocks nonselective CRAC at concentrations of 100–135 μM [24–29]. To distinguish Ca^{2+} flux through L-type channels from CRAC in OBL cells, the selective L-type calcium channel blocker verapamil was used. To our knowledge, there is no report demonstrating that verapamil inhibits CRAC [15, 30, 36, 39] but the inhibition of L-type channels in OBL cells by verapamil is well documented [5, 17–19]. Since verapamil failed to reduce Ca^{2+} influx during CRAC of OBL, this calcium flux clearly differs from a calcium entry via L-type channels.

However, considering all OBL cells that have been evaluated there was a disparity in the amount of Ca^{2+} increase during CRAC (50–1500 nM). This could be due, e.g., to differences in cell cycle, glucose, or energy supply [40] but also to different degrees of channel expression [cf. 37]. With respect to an appropriate filling of ICS, it should be interesting to compare the contribution of Ca^{2+} passing L-type channels and CRAC channels at the single cell level. However, since the mean increase in $[Ca^{2+}]_i$ during CRAC (elicited with TG) was about 300 nM (peak values) and thus comparable to other nonexcitable cell types [8, 38], it is suggested that ICS filling of OBL cells is dominated, at least in culture, by CRAC. If this is the case also *in vivo*, the spatiotemporal pattern of $[Ca^{2+}]_e$ may critically determine the filling of ICS via the suggested Ca^{2+}-dependent activation. Moreover, CRAC may be critically influenced by metal ions such as Ni^{2+} and this point deserves major attention when effects of metal implant materials in bone are investigated.

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