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Characterization of a 1,25(OH)$_2$-Vitamin D$_3$-Responsive Capacitative Ca^{2+} Entry Pathway in Rat Osteoblast-Like Cells

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Abstract We investigated the existence of a capacitative Ca^{2+} entry (CCE) pathway in ROS 17/2.8 osteoblast-like cells and its responsiveness to 1,25-dihydroxy-vitamin D$_3$ [1,25(OH)$_2$D$_3$]. Depletion of inner Ca^{2+} stores with thapsigargin or 1,25(OH)$_2$D$_3$ in the absence of extracellular Ca^{2+} transiently elevated cytosolic Ca^{2+} ($[Ca^{2+}]_i$); after recovery of basal values, Ca^{2+} re-addition to the medium markedly increased Ca^{2+} entry, reflecting pre-activation of a CCE pathway. Recovery of the Ca^{2+} overshoot that followed the induced CCE was mainly mediated by the plasma membrane Ca^{2+}-ATPase. Addition of 1,25(OH)$_2$D$_3$ to the declining phase of the thapsigargin-induced CCE did not modify further $[Ca^{2+}]_i$, indicating that steroid activation of CCE was dependent on store depletion. Pre-treatment with 1 μM Gd^{3+} inhibited 30% both thapsigargin- and 1,25(OH)$_2$D$_3$-stimulated CCE, whereas 2.5 μM Gd^{3+} was required for maximal inhibition (~ 85%). The activated CCE was permeable to both Mn^{2+} and Sr^{2+}. Mn^{2+} entry sensitivity to Gd^{3+} was the same as that of the CCE. However, 1-μM Gd^{3+} completely prevented capacitative Sr^{2+} influx, whereas subsequent Ca^{2+} re-addition was reduced only 30%. These results suggest that in ROS 17/2.8 cells CCE induced by thapsigargin or 1,25(OH)$_2$D$_3$ is contributed by at least two cation entry pathways: a Ca^{2+}/Mn^{2+} permeable route insensitive to very low micromolar (1 μM) Gd^{3+} accounting for most of the CCE and a minor Ca^{2+}/Sr^{2+}/Mn^{2+} permeable route highly sensitive to 1 μM Gd^{3+}. The Ca^{2+}-mobilizing agonist ATP also stimulated CCE resembling the Ca^{2+}/Sr^{2+}/Mn^{2+} permeable entry activated by 1,25(OH)$_2$D$_3$. The data demonstrates for the first time, the presence of a hormone-responsive CCE pathway in an osteoblast cell model, raising the possibility that it could be an alternative Ca^{2+} influx route through which osteotropic agents influence osteoblast Ca^{2+} homeostasis. J. Cell. Biochem. 86: 678–687, 2002.

Key words: capacitative Ca^{2+} entry; Ca^{2+} influx; Ca^{2+} regulation; 1,25-(OH)$_2$-vitamin D$_3$

Modulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in osteoblasts appears to play a role not only in their responsiveness to the action of osteotropic hormones, but also in the synergistic and antagonistic effects between circulating hormones on these cells [Said Ahmed et al., 2000]. As in most non-excitable cells [Putney and Bird, 1993; Clapham, 1995], receptor-activated Ca^{2+} signaling in osteoblasts involves two phases: transient Ca^{2+} release from inner IP$_3$-sensitive stores and a more prolonged phase of Ca^{2+} entry from the outside through Ca^{2+} channels in the plasma membrane [Said Ahmed et al., 2000]. It has been proposed that in bone cells of the osteoblast lineage, the influx of Ca^{2+} from the outside may act in concert with, and in response to, micro-environmental stimuli (i.e., mechanical forces, hormonal signals) to regulate not only cell metabolism and function within the short term,

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but also transcriptional rates of diverse genes involved in both osteoblast function and differentiation, and paracrine signaling between bone-forming osteoblasts and bone-resorbing osteoclasts at local sites of bone remodeling [Van Leeuwen et al., 1990; Duncan et al., 1998]. Voltage-dependent Ca^{2+} channels (VDCCs) particularly those from the L-type, are well described in osteoblastic cells and account for most of the sustained Ca^{2+} influx phase frequently observed in the Ca^{2+} response of these cells to many hormonal stimuli linked to activation of the PLCβ/IP_3 pathway [Duncan et al., 1998; Farach-Carson and Ridall, 1998]. In non-excitable cells, the capacitative Ca^{2+} entry (CCE) pathway (CCE or SOCE, i.e., store-operated Ca^{2+} entry, will be indistinctly used within the context of the present study) becomes activated as a consequence of a decrease and/or depletion of the Ca^{2+} content of the lumen of the endoplasmic reticulum [Putney, 1986, 1990, 1999]. A Ca^{2+} release-activated Ca^{2+} (CRAC) influx was recently described in primary cultures of osteoblast-like cells [Wiemann et al., 1998], but endowed with permeability features better correlated with a Ca^{2+}-activated non-selective cation channel than with CRAC channels. Moreover, the responsiveness of this Ca^{2+} entry pathway to the action of osteotropic hormones known to influence osteoblast Ca^{2+} homeostasis was not evaluated.

It is well documented that osteoblast-like cells, either primary cultures or established cell lines, respond to the hormonally-active form of vitamin D_3, 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$), with a fast and sustained increment in $[Ca^{2+}]_i$, which depends on both IP_3-dependent mobilization of Ca^{2+} from the endoplasmic reticulum and cation influx from the outside through L-type VDCCs [Lieberherr, 1987; Caffrey and Farach-Carson, 1989; Civitelli et al., 1990]. However, VDCCs may not represent the sole Ca^{2+} entry route in osteoblasts upon $1,25(OH)_2D_3$ stimulation, as it was shown that although these channels constitute a major steroid-activated calcium influx pathway in ROS17/2.8 cells, they account for upto 70% of the $1,25(OH)_2D_3$-dependent calcium influx [Lieberherr, 1987]. We have recently shown that in avian skeletal muscle cells, another well-recognized target for $1,25(OH)_2D_3$ non-genomic actions [Boland et al., 1995], $1,25(OH)_2D_3$ stimulates a CCE, which contributes, altogether with VDCC-mediated Ca^{2+} influx, to the overall Ca^{2+} response to the hormone [Vazquez et al., 1997, 1998]. The notion was then anticipated by us that sterol-induced activation of CCE could represent a more general, previously underestimated way, by which the steroid non-genomically modulates Ca^{2+} fluxes in other target tissues as well. In the present work, we provide evidence for the existence of a $1,25(OH)_2D_3$-responsive store-operated, CCE route in the rat osteosarcoma-derived osteoblast-like cell line ROS 17/2.8, and propose this as an alternative Ca^{2+} influx pathway through which osteotropic agents may influence osteoblast Ca^{2+} homeostasis.

MATERIALS AND METHODS

Materials

Fura-2/pentaacetoxymethyl ester (Fura-2/AM), nifedipine, verapamil, Ham's F12 medium, thapsigargin, ATP, $CaCl_2$, $SrCl_2$, $BaCl_2$, $MnCl_2$, and $GdCl_3$ were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Nutrientes Celulares (Buenos Aires, Argentina). All other reagents used were of analytical grade.

Cell Culture

ROS 17/2.8 osteoblast-like cells (rat osteosarcoma-derived) were cultured as monolayers onto glass coverslips at 37°C in Ham's F12 medium containing 10% FBS under humidified air (5.5% CO_2).

Intracellular Calcium Measurements

Intracellular Ca^{2+} changes were monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2 as previously described [Vazquez et al., 1997, 1998]. Cells grown onto glass coverslips were incubated in buffer A containing (in mM): 138 NaCl, 5 KCl, 1 $MgCl_2$, 5 glucose, 10 HEPES (pH 7.4), 1.5 $CaCl_2$, plus 0.1% bovine serum albumin (BSA), and 2 μM of the pentaacetoxymethylester derivative (membrane permeable) Fura-2/AM, in the dark during 30 min at room temperature (20–25°C) in order to minimize dye compartmentalization. Unloaded dye was washed out, and cells were stored in buffer B (buffer A without BSA and Fura-2/AM) in the dark (room temperature) by at least 10–15 min prior to use, to allow for complete intracellular dye deesterification. For fluorescence measurements, the coverslips containing dye-loaded cells were then mounted on the stage of an

inverted microscope (Nikon Diaphot 200) and maintained at 25–30°C. The excitation wavelength was switched over 340 and 380 nm employing a dual excitation monochromator from an SLM-Aminco 8100 spectrofluorimeter connected to the epifluorescence port of the microscope through an optic fiber. Emitted cellular fluorescence was collected at 510 nm and ratios from short and long wavelength signals were obtained (R = 340/380), thus making the measurement independent of variations in cellular dye content, dye leakage or photobleaching. Typical basal $[Ca^{2+}]_i$ in ROS 17/2.8 cells were 110 ± 5 nM. Mn^{2+} influx was assayed as the increment in Fura-2 fluorescence quenching by the cation entering the cell measured at 360 nm (the Ca^{2+}-independent, isoemissive wavelength for Fura-2). The data are expressed as the ratio of Fura-2 fluorescence due to excitation at 340 nm to that due to excitation at 380 nm. Ca^{2+}-free extracellular medium means free $[Ca^{2+}]_i$ below 10 nM, which is accomplished by preparing a nominally Ca^{2+} free buffer B (see composition above) with buffers and saline solutions being prepared in deionized water. In order to avoid chelation of Mn^{2+} or Gd^{3+}, the use of EGTA in the incubation media was avoided, because these cations bind EGTA with high affinity. Free Ca^{2+} levels were calculated by using the WinMaxc program, version 1.7-1996 [Bers et al., 1996]. The ionic strength of buffer B calculated according to Harrison and Bers [1987] was 0.147 M. Both nifedipine (5 μM) and verapamil (5 μM) were included in all incubating media in order to inhibit Ca^{2+} influx through VDCCs. This allows for functional isolation of CCE even in the presence of VDCCs [Vazquez et al., 1998; Broad et al., 1999].

$1,25(OH)_2D_3$ and thapsigargin were used dissolved in ethanol and dimethyl sulfoxide (DMSO), respectively (vehicle final concentration was less than 0.1%).

RESULTS AND DISCUSSION

The CCE pathway in ROS 17/2.8 cells was evaluated by using the Ca^{2+} re-addition protocol after depleting endogenous Ca^{2+} stores with the specific sarco/endoplasmic Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin. This has been shown to be a sensitive way to fluorimetrically monitor changes in Ca^{2+} influx through the SOC entry pathway [Vazquez et al., 1998 and references therein]. Functional isolation of a SOCE pathway in cells expressing VDCCs requires suppression of the large Ca^{2+} influx that normally occurs through these channels [Vazquez et al., 1998]. We achieved this by adding nifedipine and verapamil into the incubation medium, at concentrations known to effectively block VDCC-mediated Ca^{2+} influx in ROS 17/2.8 cells [Caffrey and Farach-Carson, 1989; Khoury et al., 1995]. Although, not significant differences were observed in the magnitude of CCE irrespectively of the presence of VDCC inhibitors (not shown), all of the following experiments were made under these conditions. Cells loaded with Fura-2 were treated with thapsigargin (500 nM) in the absence of Ca^{2+} in the extracellular medium. At this concentration, thapsigargin induces effective, passive store depletion through a highly selective action on the SERCA without undesirable membrane side effects as it is known that at the concentrations typically used to rapidly inhibit SERCAs in living cells (1 μM or higher) thapsigargin has many additional, unspecific effects, some of them involving alteration of the plasma membrane cation permeability [see for instance, Taylor and Broad, 1998]. Once the rapid and transient elevation in $[Ca^{2+}]_i$ consequence of store depletion through the leak channel [Pozzan et al., 1994] occurred, Ca^{2+} re-addition (1.5 mM) was performed after $[Ca^{2+}]_i$ fell down to levels equal or close to basal (3–5 min after the peak response was reached). At this stage, it is assumed that all of the plasma membrane Ca^{2+} channels, which could have been activated by transient increases in bulk cytosolic Ca^{2+} levels or an increment in the Ca^{2+} content at the channel mouth microdomain, derived from either mobilization and/or depletion of Ca^{2+} from inner stores (i.e., Ca^{2+}-activated Ca^{2+} channels) have returned to basal activity [Barritt, 1998]; thus, if re-addition of Ca^{2+} to the extracellular millieu results in increased Ca^{2+} entry, it should exclusively reflect the activity of Ca^{2+} channels whose activation is the result of their ability to sense the filling state of store Ca^{2+} content, i.e., CCE. As shown in Figure 1A, re-addition of Ca^{2+} after recovery from the thapsigargin Ca^{2+} response resulted in a fast $[Ca^{2+}]_i$ rise (about 2–2.5-fold over basal) evidencing Ca^{2+} influx from the outside through a pre-activated CCE pathway. Ca^{2+} re-admission to cells not previously exposed to thapsigargin resulted in no detectable changes in Ca^{2+} levels; also treatment of cells with vehicle DMSO alone did not affect

neither basal intracellular Ca^{2+} levels nor CCE activity (data not shown). As previously reported [Civitelli et al., 1990], stimulation of ROS 17/2.8 cells with 10^{-8} M $1,25(OH)_2D_3$ in Ca^{2+}-free medium resulted in a rapid (~30 s), but transient $[Ca^{2+}]_i$ rise (Fig. 1B) entirely due to mobilization from endogenous stores, which correlates well with the sterol-dependent generation of IP_3 in these cells, thus indicating that release of Ca^{2+} from the IP_3-responsive store occurred. Moreover, it was proven that $1,25(OH)_2D_3$ mobilized Ca^{2+} from thapsigargin sensitive pools, as pre-treatment of the cells with the SERCA inhibitor totally abolished the response to the hormone; in addition, treatment of cells with thapsigargin after the $1,25(OH)_2D_3$-induced Ca^{2+} transient mobilization occurred resulted in poor additional Ca^{2+} release, indicating that the sterol almost completely depleted endogenous stores (not shown). We next evaluated the ability of the sterol to activate the SOCE pathway in ROS 17/2.8 cells by using the above-mentioned experimental protocol. Similarly to what occurred with thapsigargin, Ca^{2+} re-addition after the Ca^{2+} transient induced by $1,25(OH)_2D_3$ took place resulted in a fast increase in cytosolic Ca^{2+} revealing activation of CCE (Fig. 1B). Treatment of cells with vehicle (ethanol $< 0.1\%$) resulted in no changes of neither basal intracellular Ca^{2+} levels nor CCE activity. The specificity of the effects of $1,25(OH)_2D_3$ compared to other steroids and parathyroid hormone was evidenced by the fact that addition of either 17β-estradiol, progesterone, or PTH (10^{-8} and 10^{-9} M) caused no changes of $[Ca^{2+}]_i$. However, these observations do not exclude the possibility that related metabolites, for example, $24,25(OH)_2D_3$ or $25(OH)D_3$, might also influence the SOCE pathway. In ROS 17/2.8 cells, $24,25(OH)_2D_3$ has been shown to reduce or even inhibit $1,25(OH)_2D_3$-dependent activation of L-type calcium channels, although through a mechanism apparently involving a receptor other than that for $1,25(OH)_2D_3$ signaling [Takeuchi and Guggino, 1996]; additionally, it has been observed that $25(OH)D_3$ reproduced $1,25(OH)_2D_3$ actions on calcium release in these cells [Civitelli et al., 1990], its effects on the calcium entry phase being less significant. Future studies should address possible modulatory effects of the different vitamin D-derived steroids on the $1,25(OH)_2D_3$-responsive SOCE pathway.

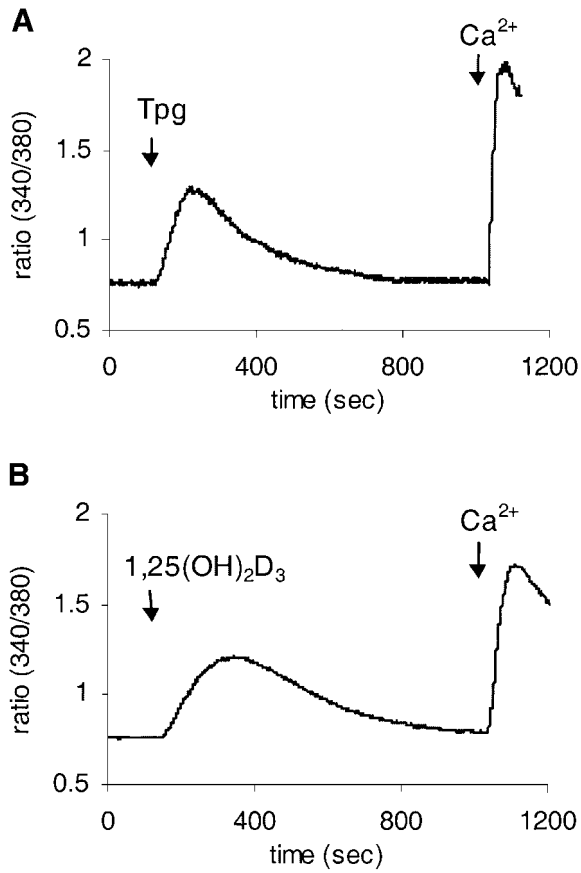

Fig. 1. Thapsigargin and $1,25(OH)_2D_3$ induced CCE in ROS 17/2.8 osteoblast-like cells. Fura-2-loaded osteoblasts were incubated in Ca^{2+}-free medium containing 5 μM nifedipine and 5 μM verapamil, in order to block Ca^{2+} influx through VDCCs; this and all other experiments in the present article were performed under these conditions, to allow for functional isolation of CCE even in the presence of VDCCs (see text for comments and references). In (**A**), inner stores were depleted by inhibiting the SERCAs with 500 nM thapsigargin (Tpg). After cytosolic Ca^{2+} returned to basal levels, Ca^{2+} (1.5 mM) was re-added to the medium to evidence the existence of a CCE pre-activated pathway. (**B**) Cells were treated with 10^{-8} M $1,25(OH)_2D_3$; once the hormone-induced Ca^{2+} transient occurred, Ca^{2+} (1.5 mM) was re-added to the medium. Shown are time traces representative from at least five independent experiments, each performed onto no less than 40–50 cells.

In both thapsigargin- or $1,25(OH)_2D_3$-induced CCE, once the maximum level of Ca^{2+} entry was achieved, the Ca^{2+} overshoot decayed with a kinetics highly comparable to the declining phase, which followed the initial store-dependent Ca^{2+} transient of either the thapsigargin- or sterol-dependent Ca^{2+} response, suggesting that plasma membrane Ca^{2+} ATPase (PMCA) was likely the main mechanism of Ca^{2+} extrusion, as established in other cell systems [Snitsarev and Taylor, 1999]. In fact, omitting

Na^+ in the extracellular medium (iso-osmotically replaced by Li^+) did not affect neither the Ca^{2+} overshoot following store depletion nor the kinetics of its decaying phase, thus ruling out the participation of the Na^+/Ca^{2+} exchanger as an extruding mechanism or at least putting its contribution into an insignificant level (Fig. 2). A slight reduction in the amplitude of both the Ca^{2+} overshoot and its recovery phase, but not its kinetics, could be attributed to a minor unspecific effect of Li^+, as this observation was not made when Na^+ was reduced or even eliminated without replacement by Li^+ (not shown). Adding $1,25(OH)_2D_3$ to the declining phase of the thapsigargin-induced CCE (Fig. 3) did not modify further Ca^{2+} levels, thus indicating that sterol-dependent activation of SOC channels was indeed triggered by a decrease in the luminal Ca^{2+} content of endogenous stores, and not by the effect of a signal transducer element acting upstream to store depletion. This is of importance because it is known that $1,25(OH)_2D_3$-dependent stimulation of the PLC/IP_3 cascade in both primary-cultured as well as osteosarcoma-derived rat osteoblasts involves activation of a PLCβ-coupled, pertussis toxin-insensitive Gq protein [Farach-Carson et al., 1991; Le Mellay et al., 1997, 1999; Farach-Carson and Ridall, 1998], and it has been reported that Gq-coupled pathways play a role in store-independent activation of some classes of SOC channels [Zhu et al., 1998; Boulay et al., 1997].

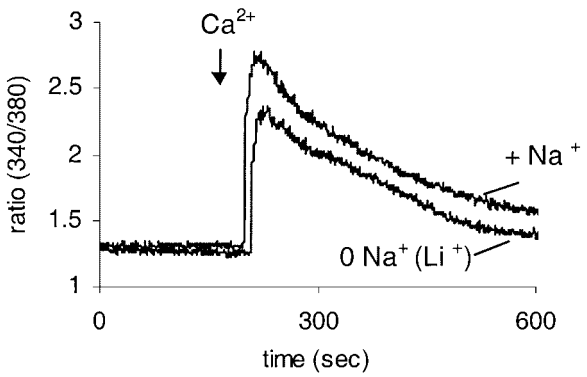

Fig. 2. Plasma membrane Ca^{2+}-ATPase (PMCA) is the major extrusion component involved in the recovery phase of CCE in osteoblasts. Fura-2-loaded osteoblasts were incubated in Ca^{2+}-free medium and inner stores were depleted with 500 nM thapsigargin (see **panel A** in Fig. 1). After cytosolic Ca^{2+} returned to basal levels (for simplification, the time-trace curves are shown from hereon), Ca^{2+} (1.5 mM) was re-added to the medium. As indicated, the recovery phase of CCE was monitored in either normal extracellular Na^+ ($[Na^+]_o$ = 138 mM) or in the absence of extracellular Na^+ ($[Na^+]_o = 0$, but replaced in equimolar amounts by Li^+) to inhibit the Na^+/Ca^{2+}-exchanger. Shown are time traces representative from at least two independent experiments, each performed onto no less than 40 cells.

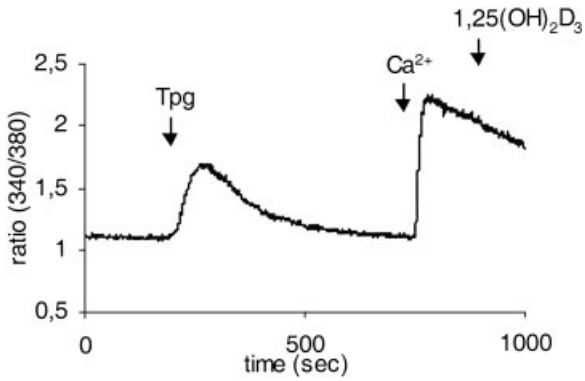

Fig. 3. In ROS 17/2.8 cells the $1,25(OH)_2D_3$-induced CCE is not independent of store depletion. Fura-2-loaded osteoblasts were incubated in Ca^{2+}-free medium and inner stores were depleted with 500 nM thapsigargin (Tpg). After cytosolic Ca^{2+} returned to basal levels, Ca^{2+} (1.5 mM) was re-added to the medium and the CCE allowed to proceed. Before reaching basal levels, 10^{-8} M $1,25(OH)_2D_3$ was added to test for additional steroid-dependent CCE in the presence of emptied stores (see text). Shown are time traces representative from at least three independent experiments, each performed onto no less than 40 cells.

Within the low micromolar range, Gd^{3+} has been shown to be a selective blocker of CCE, thus becoming an useful pharmacological tool, particularly in cells in which different Ca^{2+} entry pathways co-exist [see for instance Broad et al., 1999]. Pre-treating ROS 17/2.8 osteoblast-like cells with low micromolar concentrations of Gd^{3+} dose-dependently inhibited both thapsigargin- and $1,25(OH)_2D_3$-stimulated CCE with no significant effect on the Ca^{2+} release phase (Fig. 4). At 1-μM Gd^{3+}, CCE was reduced about 25–30% respect to control cells not treated with Gd^{3+}, whereas concentrations of the lanthanide equal or higher than 2.5 μM were required to achieve maximal inhibition (~85%). The small Ca^{2+} entry remaining at concentrations of Gd^{3+} as high as 10 μM could be reflecting either a CCE route insensitive to Gd^{3+} or a population of non-CCE, Ca^{2+}-activated Ca^{2+} channels, which did not fully recover their basal status at the time of Ca^{2+} re-addition to the medium.

In various cell types, the CCE pathway permeates Ba^{2+}, Mn^{2+}, and/or Sr^{2+}; thus, these cations could be used as Ca^{2+} surrogates by monitoring their effects on the fluorescence of

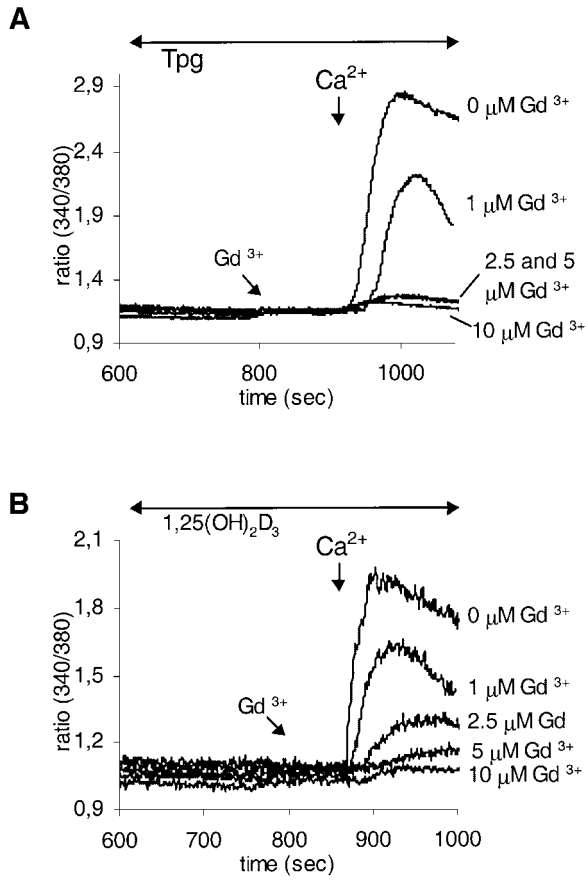

Fig. 4. Gd^{3+} blockade of the thapsigargin- and $1,25(OH)_2D_3$-induced CCE in ROS 17/2.8 cells. Fura-2-loaded osteoblasts incubated in Ca^{2+}-free medium were treated with (**A**) 500 nM thapsigargin (Tpg) or (**B**) 10^{-8} M $1,25(OH)_2D_3$. Once the transient Ca^{2+} rise that followed store depletion by either of these two Ca^{2+} mobilizing agents occurred, Gd^{3+} (arrowhead) was added at the indicated concentrations, and 2 min later, Ca^{2+} (1.5 mM) was re-added to the medium. Shown are time traces representative from at least four independent experiments, each performed onto no less than 40 cells.

cytosolic Fura-2. Both Ba^{2+} and Sr^{2+} produce an excitation spectrum and maximal brightness highly similar to Ca^{2+}, whereas Mn^{2+} quenches Fura-2 fluorescence [Kwan and Putney, 1990]. Neither Ba^{2+} nor Mn^{2+} can be removed from cytosol because they are not substrates for neither SERCAs nor PMCAs [Byron and Taylor, 1995], thus these two cations offer a reliable way to monitor unidirectional entry of bivalent cations through the CCE pathway. In ROS 17/2.8 cells, re-addition of Ba^{2+} (1.5 mM) after the cells were incubated in Ca^{2+}-free medium, resulted in a significant cation influx (30–40% over basal) reflecting basal permeability of the plasma membrane to Ba^{2+}. Unlike what occurs in other cell types [Zhu et al., 1998], the rate of Ba^{2+} entry was not further increased by thapsigargin-induced store depletion, indicating that the CCE pathway was not responsible for Ba^{2+} permeation into osteoblastic cells (not shown). However, the capacitative cation entry stimulated by either thapsigargin or $1,25(OH)_2D_3$ was highly permeable to Mn^{2+}, reflected by Mn^{2+}-induced quenching of Fura-2 fluorescence in response to either agent (Fig. 5A), as is the case for several cell types where store-operated cation influx pathways have been described [Putney and Bird, 1993; Fasolato et al., 1994; Berridge, 1995]. Sensitivity to Gd^{3+} of thapsigargin-dependent Mn^{2+} entry closely resembled that of the CCE pathway (Fig. 5B), suggesting that the same cation entry route—at least on a pharmacological basis—mediated both Ca^{2+} and Mn^{2+} influx into the cell. It should be noted here that at the Gd^{3+} concentration allowing for maximal blockade of the thapsigargin-dependent CCE (2.5 μM Gd^{3+}, see Fig. 4), Mn^{2+} influx was also completely abolished, but without residual cation permeation being detectable. This reinforces the above-stated interpretation that the remaining Ca^{2+} entry seen under conditions at which CCE was completely abolished, represents most likely residual activity of non-CCE (non-Mn^{2+} permeable), Ca^{2+}-activated Ca^{2+} channels.

As shown in Figure 6, the thapsigargin-stimulated capacitative cation entry was also permeable to the Ca^{2+} surrogate Sr^{2+}, although, the magnitude of Sr^{2+} influx following store depletion was significantly lower (approximately threefold) than that when Ca^{2+} was the cation re-added instead (not shown). After peaking, Sr^{2+} entry was followed by a rapid decay to pre-stimulation levels, in line with the fact that this cation can be handled by PMCAs [Broad et al., 1999]. Store-depletion activated Sr^{2+} entry did not affect the subsequent influx of Ca^{2+}. Moreover, pre-treatment of the cells with 1 μM Gd^{3+} completely prevented capacitative Sr^{2+} influx, whereas subsequent Ca^{2+} re-addition was reduced in only 30% respect to non-Gd^{3+} treated, control cells (Fig. 6). Similar results were obtained by inducing store-depletion with $1,25(OH)_2D_3$ (not shown). Thus, the Sr^{2+} permeable capacitative pathway strongly resembled the Ca^{2+}/Mn^{2+} permeable component of the overall CCE, which was sensitive to 1 μM Gd^{3+}. Taken together, these results suggest that in ROS 17/2.8 osteoblast-like cells,

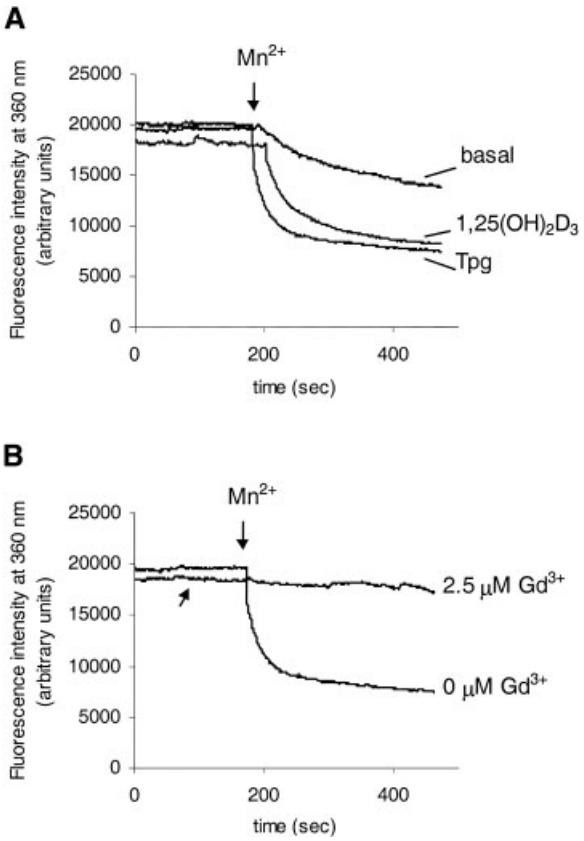

Fig. 5. Mn^{2+} permeability of the thapsigargin- and $1,25(OH)_2D_3$-induced CCE in ROS 17/2.8 osteoblast-like cells. **(A)** Fura-2-loaded osteoblasts incubated in Ca^{2+}-free medium were treated with either 500 nM thapsigargin (Tpg) or 10^{-8} M $1,25(OH)_2D_3$. Once the transient Ca^{2+} rise that followed store depletion by either of these two Ca^{2+} mobilizing agents occurred (time-trace curves are shown from hereon), Mn^{2+} (100 μM) was incorporated into the medium and its entry was monitored as the quenching of Fura-2 fluorescence measured at an excitation wavelength of 360 nm. **(B)** Blockage of thapsigargin-induced Mn^{2+} influx by 2.5 μM Gd^{3+} added (arrowhead) 2 min before Mn^{2+}. Shown are time traces (fluorescence intensity at 360 nm) representative from at least three independent experiments, each performed onto no less than 40 cells.

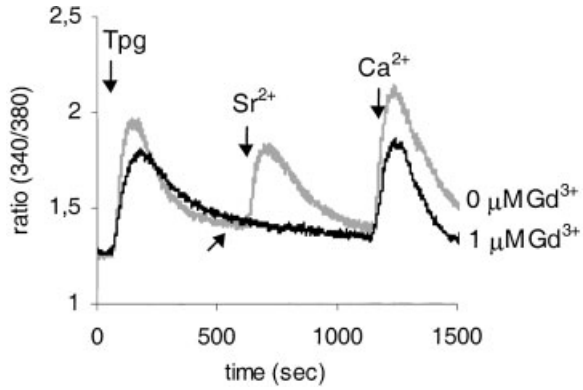

Fig. 6. Sr^{2+} permeability of the thapsigargin-induced CCE in ROS 17/2.8 cells. Fura-2-loaded osteoblasts incubated in Ca^{2+}-free medium were treated with 500 nM thapsigargin (Tpg). Once the store depletion-dependent transient Ca^{2+} rise occurred, the measurement continued in the absence (gray trace) or presence of Gd^{3+} (added at arrowhead; dark trace) at the indicated concentration, and 2 min later, Sr^{2+} (1.5 mM) was added. After full recovery from the cytosolic Sr^{2+} rise that followed Sr^{2+} influx into the cell (see text), Ca^{2+} (1.5 mM) was re-added to the incubating medium. Shown are time traces representative from at least two independent experiments, each performed onto no less than 40 cells.

the CCE induced by thapsigargin or $1,25(OH)_2D_3$ is contributed by at least two cation entry pathways: a Ca^{2+}/Mn^{2+} permeable route insensitive to very low micromolar (1 μM) Gd^{3+} accounting for most of the total CCE, and a minor—in terms of the contribution to the total CCE—$Ca^{2+}/Sr^{2+}/Mn^{2+}$ permeable route highly sensitive to very low micromolar Gd^{3+}.

Osteoblasts, as other cell types, respond to extracellular nucleotides with an elevation in cytosolic Ca^{2+} levels due to stimulation of specific purinergic membrane receptors from the P2Y type, which are coupled to the Gq/PLCβ/IP_3 pathway [Kunapuli and Daniel, 1998; Bowler et al., 1999]. ROS 17/2.8 cells respond to micromolar concentrations of either ATP or UTP, but not ADP, with a strong elevation in cytosolic Ca^{2+}, subsequent to mobilization of Ca^{2+} from IP_3/thapsigargin-sensitive stores [Vazquez et al., unpublished communications], consistent with expression of the P2Y2 receptor subtype [Kunapuli and Daniel, 1998]. In order to test the possibility that CCE modulation could represent a previously underestimated way for osteotropic agonists to influence osteoblast Ca^{2+} homeostasis, we evaluated if ATP-stimulated Ca^{2+} mobilization from inner stores in ROS 17/2.8 cells resulted, as was the case for the steroid $1,25(OH)_2D_3$, in activation of CCE. Stimulation of osteoblasts with 10-μM ATP in Ca^{2+}-free medium resulted in a typical ATP-driven Ca^{2+} response, composed by a fast and transient cytosolic Ca^{2+} rise, which rapidly declined to pre-stimulating levels (Fig. 7A). As for the steroid, pre-treatment of the cells with thapsigargin completely abolished the response to ATP (not shown). Re-adding Ca^{2+} after cytosolic levels reached basal values evidenced the existence of an activated CCE pathway, whose kinetics was comparable to that from the thapsigargin- and $1,25(OH)_2D_3$-induced CCE,

but of significantly lower amplitude (~30% over basal). The capacitative pathway stimulated by ATP was also permeable to Sr^{2+} (Fig. 7B), and pre-treatment with 1 μM Gd^{3+} completely abolished either Ca^{2+} or Sr^{2+} entry induced by the purinergic agonist (Fig. 7A,B). These results suggest that the ATP-induced CCE pathway in ROS17/2.8 cells corresponds to the Ca^{2+}/Sr^{2+}/Mn^{2+} permeable (highly sensitive to very low micromolar Gd^{3+}) non-selective cation entry pathway, which becomes activated as part of the total CCE stimulated by either thapsigargin or $1,25(OH)_2D_3$ (see above). As Ca^{2+} entry through the SOCE pathway is proportional to the degree of depletion of the intracellular Ca^{2+} store [Vazquez et al., 1998, and references therein], the possibility arises that the ability of ATP to stimulate only one of the two sets of CCE channels here characterized might obey to a lower Ca^{2+} mobilization from the IP_3-sensitive store by the purinergic agent compared to the more robust Ca^{2+} depletion induced by either thapsigargin or $1,25(OH)_2D_3$. In fact, thapsigargin addition to cells, which recovered from the ATP-stimulated IP_3-induced Ca^{2+} transient resulted in a significant additional release of Ca^{2+} from stores (not shown), thus evidencing that Ca^{2+} mobilization through the purinergic pathway was partial. This speculative interpretation is plausible only if the assumption is made that CCE channels might exist bearing differential sensing capabilities in order to respond to different degrees of decrease in luminal Ca^{2+} content of the stores. This notion implicitly includes the concept of an all-or-none response of certain CCE channels depending on the extent of store depletion. Evidence indicating that this could be the case has been recently provided [Parekh et al., 1997; Huang and Putney, 1998].

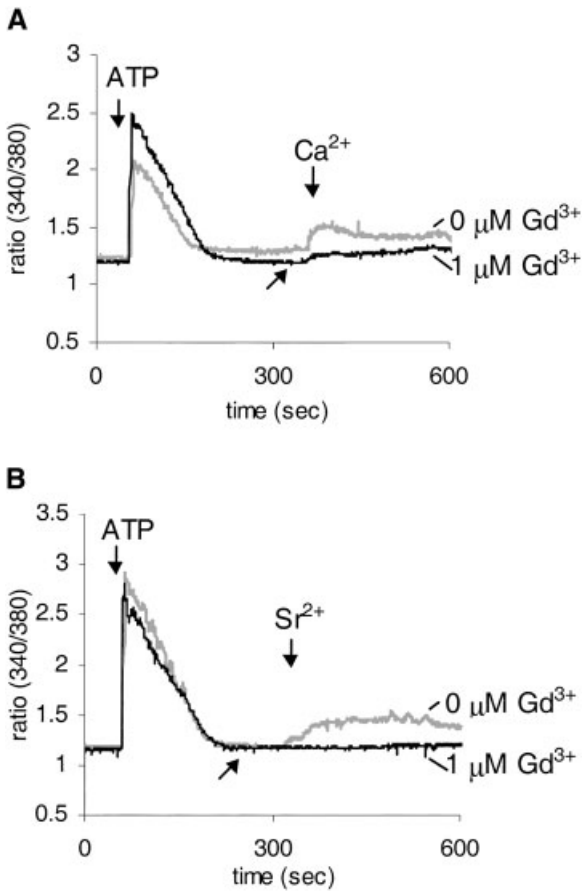

Fig. 7. ATP-dependent CCE in ROS 17/2.8 osteoblast-like cells. Fura-2-loaded osteoblasts were incubated in Ca^{2+}-free medium, and when indicated, exposed to 10 μM ATP. The transient Ca^{2+} rise that followed ATP treatment corresponded to the ATP-induced IP_3-mediated release of Ca^{2+} from inner stores. After cytosolic Ca^{2+} returned to basal, the measurement continued in the absence (gray trace) or presence of 1 μM Gd^{3+} (added at arrowhead; dark trace), and 2 min later, either Ca^{2+} (1.5 mM, **panel A**) or Sr^{2+} (1.5 mM, **panel B**) were incorporated into the medium. Shown are time traces representative from at least three independent experiments, each performed onto no less than 40 cells.

The present studies represent, to our knowledge, the first evidence to date reporting the existence of CCE in an osteoblast-like cell model, and rise the possibility that this could be an alternative Ca^{2+} influx pathway through which osteotropic agents can influence osteoblast Ca^{2+} homeostasis. Both the secosteroid $1,25(OH)_2D_3$ and the purinergic agonist ATP, which are well known to act on osteoblasts stimulating either the PLC/IP_3/DAG pathway, release of Ca^{2+} from inner stores and Ca^{2+} influx through L-type VDCCs [Farach-Carson and Ridall, 1998; Bowler et al., 1999], have been shown here to be able to differentially stimulate two pharmacologically discernible CCE. It is likely that under physiological conditions, at which osteoblasts are exposed to the action of more than one hormone at a time, Ca^{2+} enters through multiple Ca^{2+}-permeant CCE routes, the relative contribution of each probably depending on the type of receptor pathway activated—i.e., on the type of osteotropic agent—the degree of stimulation—i.e., the agonist concentration, and the effect of compensatory, counteracting signaling pathways—and ultimately, on the state of differentiation of the osteoblast. Within this context, the present findings set the basis for future studies focused on the

characterization of the regulatory signaling pathways underlying the activation of the osteoblast CCE by either $1,25(OH)_2D_3$, ATP, or other hormones, which could potentially activate CCE as part of their strategy to modulate the osteoblast Ca^{2+} homeostasis. Also, additional studies using other osteoblastic cell culture models are necessary before arriving at the conclusion that hormone-dependent modulation of the CCE pathway is a mechanism applicable to osteoblasts in general.

Finally, at least seven mammalian genes designated TRP1 through TRP7 [Birnbaumer et al., 1996; Putney, 1999] are currently known, whose heterologous expression in diverse cell lines results most often in appearance of non-selective cation channels, some of them being candidates for mediating CCE, particularly when such CCE involves a non-selective cation pathway. Recently, TRP homologues have been cloned from a rat brain cDNA library [Mizuno et al., 1999], the expression of two of them (rTRP3 and rTRP6) into COS cells resulting in increased store-operated Ca^{2+} entry. If the CCE here described in osteoblasts is related to the expression of some of these TRP proteins is under current investigation.

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