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Evidence on the Operation of ATP-Induced Capacitative Calcium Entry in Breast Cancer Cells and Its Blockade by 17β-Estradiol

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Abstract Little is known about the regulation of cytosolic calcium Ca^{2+} levels ($[Ca^{2+}]_i$) in breast cancer cells. We investigated the existence of capacitative calcium entry (CCE) in the tumorigenic cell line MCF-7 and its responsiveness to ATP. MCF-7 cells express purinergic receptors as well as estrogen receptors (ER). Depletion of calcium stores with thapsigargin (TG, 500 nM) or ATP (10 μM) in the absence of extracellular Ca^{2+}, resulted in a rapid and transient elevation in $[Ca^{2+}]_i$. After recovery of basal levels, Ca^{2+} readmission (1.5 mM) to the medium increased Ca^{2+} influx (twofold over basal), reflecting pre-activation of a CCE pathway. Cells pretreated with TG were unable to respond to ATP, thus indicating that the same Ca^{2+} store is involved in their response. Moreover, IP_3-dependent ATP-induced calcium mobilization and CCE were completely blocked using compound U-73122, an inhibitor of phospholipase C. Compound 2-APB (75 μM) and Gd^{3+} (10 μM), antagonists of the CCE pathway, completely prevented ATP-stimulated capacitative Ca^{2+} entry. CCE in MCF-7 cells was highly permeable to Mn^{2+} and to the Ca^{2+} surrogate Sr^{2+}. Mn^{2+} entry sensitivity to Gd^{3+} matched that of the Ca^{2+} entry pathway. 17β-estradiol blocked ATP-induced CCE, but was without effect on TG-induced CCE. Besides, the estrogen blockade of the ATP-induced CCE was completely abolished by preincubation of the cells with an ER monoclonal antibody. ER α immunoreactivity could also be detected in a purified plasma membrane fraction of MCF-7 cells. These results represent the first evidence on the operation of a ATP-responsive CCE pathway in MCF-7 cells and also indicate that 17β-estradiol interferes with this mechanism by acting at the cell surface level. J. Cell. Biochem. 87: 324–333, 2002.

Key words: breast cancer cells; capacitative Ca^{2+} entry; ATP; 17β-estradiol; non-genomic effects

Agonist-dependent regulation of intracellular calcium concentration ($[Ca^{2+}]_i$) in most cell types involves modulation of either Ca^{2+} release from inner stores through activation of the phospholipase C (PLC)-inositol trisphosphate (IP_3) cascade, or Ca^{2+} entry through Ca^{2+} channels in the plasma membrane, or both [Tsien and Tsien, 1990; Putney and Bird, 1993; Berridge, 1995; Clapham, 1995]. In addition, Ca^{2+} release from intracellular pools also triggers Ca^{2+} influx across the plasma membrane, a process known as capacitative calcium entry (CCE) [Putney, 1997]. The exact mechanism by which intracellular Ca^{2+} release is coupled to extracellular Ca^{2+} entry is still uncertain, but the starting event is somehow derived from depletion of the endoplasmic reticulum calcium content [Putney, 1997]. Two hypotheses prevail: conformational coupling [Berridge, 1995] and the participation of a diffusible signal released from the stores [Randriamampita and Tsien, 1993]. The mechanisms that control $[Ca^{2+}]_i$ are closely linked to many cellular processes such as proliferation, differentiation, secretion, metabolism, and apoptosis. Little is known about cytosolic Ca^{2+} regulation in breast cancer cells, although this knowledge would be useful to elucidate part of the mechanism activated during carcinogenesis.

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Breast cancer MCF-7 cells have been reported to express purinergic receptors from the $P2Y_2$ subtype (that can be activated by ATP), which couple to PLC through heterotrimeric G-proteins [Flezar and Heisler, 1993; Ralevic and Burnstock, 1998]. This cell line also expresses estrogen receptors (ER) and has proved to be a useful model to evaluate estradiol effects on tumorigenic mammary cells. The classical ER α locates almost exclusively in the nucleus, but studies on fast estrogen non-genomic effects have suggested that the receptor may also be associated to the cell surface [Graber et al., 1993; Aronica et al., 1994; Migliaccio et al., 1996]. In other cell types several authors have provided solid support to the idea that a parallel reservoir of ER or ER-related proteins resides in the plasma membrane and causes very rapid signal transduction events [Luconi et al., 1999; Razandi et al., 1999; Norfleet et al., 2000; Hall et al., 2001; Adams et al., 2002; Chambliss and Shaul, 2002]. In MCF-7 cells, cell surface localization of ER α has been evidenced by conventional immunocytochemistry, confocal microscopy, and competition of [^{3}H]17β-estradiol binding to intact cells by impeded estrogen conjugates [Monje et al., 2001].

To further characterize the mechanisms that control $[Ca^{2+}]_i$ in MCF-7 cells, we have investigated the existence of a CCE pathway and its responsiveness to ATP. Moreover, in view of the evidence on the existence of plasma membrane-associated ER and the role that the Ca^{2+} messenger system may play in the proliferative effects of estrogen, we have also studied whether 17β-estradiol influences ATP modulation of intracellular Ca^{2+} regulation in these cells.

MATERIALS AND METHODS

Chemicals

Fura-2/pentaacetoxymethyl ester (Fura-2/AM), nifedipine, verapamil, thapsigargin (TG), ATP and compound 2-aminoethoxydiphenyl borate (2-APB) were purchased from Sigma Chemical Co. (St. Louis, MO). Compounds U-73122 and U-73343 were from Biomol (Plymouth, PA). Fetal bovine serum (FBS) was purchased from Gen S.A. (Buenos Aires, Argentina) and RPMI-1640 medium from Hyclone (Milan, Italy). All other reagents used were of analytical grade. Anti-ER α mouse monoclonal antibody (Ab10, clone TE111.5D11, directed against the ligand-binding domain of ER) was obtained from NeoMarkers (Fremont, CA). The anti-Lamin B goat polyclonal IgG (M-20) and anti-Gαs rabbit polyclonal IgG (K-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence blot detection kit was from Amersham (Buckinghamshire, England). Molecular weight colored markers were bought from BioRad Laboratories (Richmond, CA).

Cell Culture

MCF-7 cells (a human breast cancer epithelial cell line) were obtained from the American Type Culture Collection (Rockville, MD) and kindly provided by Dr. A. Baldi (Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina). Cells were cultured in serum-supplemented media composed of RPMI-1640, 10% FBS, penicillin, and streptomycin. Confluent monolayers were harvested by trypsin (0.1%) treatment. Cultures were established and maintained at 37°C in a humid air atmosphere (5.5% CO_2). The medium was replaced every 2 days and cultures were passaged every 4–5 days. For evaluating 17β-estradiol effects, serum was removed and cells were incubated for 24 h in RPMI-1640 medium (without phenol red) containing 0.1% bovine seroalbumin (BSA). 17β-estradiol was added, dissolved in ethanol. The vehicle was applied in an equivalent concentration to control cells (less than 0.1% in all cases).

Intracellular Calcium Measurements

Intracellular Ca^{2+} changes were monitored using the Ca^{2+}-sensitive fluorescent dye Fura-2 [Vazquez et al., 1997, 1998]. Cells attached to glass coverslips were incubated in loading buffer containing (in mM): 138 NaCl, 5 KCl, 1 $MgCl_2$, 5 glucose, 10 HEPES (pH 7.4), 1.5 $CaCl_2$, plus 0.1% BSA, and 2 μM of the membrane permeable Fura-2/AM. In order to minimize dye compartmentalization, cells were loaded in the dark during 30 min at room temperature (20–25°C). Unloaded dye was washed out and cells were stored in measurement buffer (loading buffer without BSA and Fura-2/AM) in the dark (room temperature) for at least 15–20 min prior to measurement, in order to allow complete intracellular dye de-esterification. 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 from 340 to 380 nm employing a dual excitation monochromator from an SLM-Aminco 8100 spectrofluorimeter. Emitted cellular fluorescence was collected at 510 nm and ratios from short to long wavelength signals were obtained, thus making the measurement independent of variations in cellular dye content, dye leakage or photobleaching. No appreciable differences were observed when performing spectrofluorimetric measurements at 37°C. 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). Ca^{2+}-free extracellular medium means free Ca^{2+} concentration below 10 nM, which is accomplished by preparing a nominally Ca^{2+}-free measurement buffer (see composition above) with all solutions being prepared in deionized water. In order to prevent chelation of Mn^{2+} or Gd^{3+}, EGTA was not included in the incubation media, since it binds to these cations with high affinity [Sadoshima et al., 1992]. Free Ca^{2+} levels were calculated by using the WinMaxc program version 1.7 [Bers et al., 1994]. The ionic strength of the measurement buffer was 0.147 M [Harrison and Bers, 1987]. Both nifedipine (5 μM) and verapamil (5 μM) were included in all incubating media in order to inhibit Ca^{2+} influx through VDCCs. This makes possible the functional isolation of capacitative Ca^{2+} entry even in the presence of VDCCs [Vazquez et al., 1998; Broad et al., 1999]. The data are given as the ratio of fluorescence obtained at the excitation wavelengths of 340 and 380 nm (F340/F380) from groups of 10–12 cells. Resting levels of $[Ca^{2+}]_i$ in MCF-7 cells were 60–90 nM. All measurements shown are representative of at least three and, in most cases, a greater number of separate experiments.

Purification of Plasma Membranes

Purified plasma membrane (PM) preparations were obtained by a method previously reported [Suzuki et al., 1989; Kim et al., 1996] with some modifications. Cells were washed and collected in TEDK buffer solution (10 mM Tris-HCl, 0.3 M KCl, 1 mM EDTA, 5 mM dithiothreitol, pH 7.4). The cell suspension was homogenized with a teflon-glass hand homogenizer until more than 95% of the cells were disrupted (50 strokes). The homogenized solution was centrifuged at 500*g* for 10 min. The supernatant fluid was centrifuged at 27,000*g* for 30 min. The pellet was resuspended in 1.5 ml of 15% sucrose in TEDK and layered on a discontinuous sucrose density layer made up of 2 ml of 30% sucrose in TEDK layered onto 1.5 ml of 45% sucrose TEDK. This gradient was centrifuged at 76,000*g* for 3 h. The 15–30% interface (PM fraction) was collected, diluted with TEDK, and centrifuged at 105,000*g* for 1 h. The resulting PM fraction was resuspended in TEDK. The purity of this fraction was assessed by Western blot detection of the specific markers Gαs (plasma membrane) and Lamin B (nuclei) and by determination of glucose-6-P phosphatase (endoplasmic reticulum).

Western Blot Analysis

ER α immunoreactivity was analyzed in total homogenates, endoplasmic reticulum, and plasma membrane fractions. Protein samples were suspended in sample buffer (400 mM Tris/HCl pH 6.8, 10% SDS, 50% glycerol, 500 mM DTT, and 2 μg/ml bromophenol blue), and denatured for 5 min. The proteins (equal amounts) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS–PAGE). Fractionated proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore), using a semi-dry system. Nonspecific sites were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). Blots were incubated for 1 h with the appropriate dilution of the primary antiserum. The membranes were repeatedly washed with PBS-T prior to incubation with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence blot detection kit was used to see the reactivebands (Amersham, Piscataway, NJ). Relative migration of unknown proteins was determined by comparison with molecular weight markers.

RESULTS AND DISCUSSION

In order to dissociate intracellular Ca^{2+} release and Ca^{2+} influx responses, our experiments were carried out according to the calcium-free/calcium readdition protocol, which is a sensitive method to monitor calcium influx changes through CCE [Vazquez et al., 1998; Picotto et al., 1999]. In addition, the experiments were performed in the presence of nifedipine and verapamil to inhibit Ca^{2+} influx through VDCCs (see Methods). As shown in

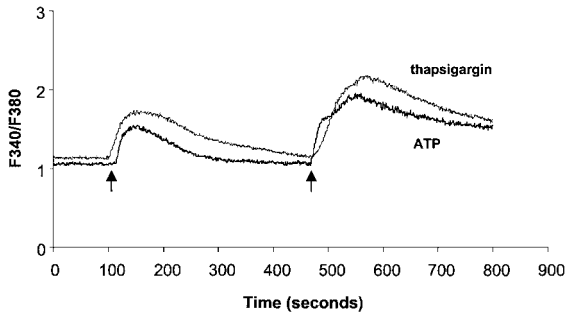

Fig. 1. Thapsigargin and ATP induce capacitative Ca^{2+} entry (CCE) in MCF-7 cells. Fura-2 loaded cells were incubated in Ca^{2+}-free medium and treated as follows: inner stores were depleted by inhibiting the SERCAs with 500 nM thapsigargin or exposed to 10 μM ATP (left arrow). After cytosolic Ca^{2+} returned to basal levels, Ca^{2+} (1.5 mM) was restored to the medium (right arrow).

Fig. 1, depletion of intracellular calcium stores with the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor TG (500 nM) in the absence of extracellular Ca^{2+}, resulted in a rapid and transient elevation of $[Ca^{2+}]_i$. When used at this dose, thapsigargin induces passive store depletion without any membrane side effects [Wiemann et al., 1998, 1999; Jan et al., 1999] and causes the selective, complete, and irreversible store depletion by unopposed Ca^{2+} leakage [Thastrup et al., 1990]. After recovery of basal levels (3–4 min), Ca^{2+} readmission (1.5 mM) to the extracellular medium resulted in increased cation influx (twofold over basal), reflecting the activity of plasma membrane channels whose activation depends on their ability to sense depletion of Ca^{2+} from the intracellular stores, as is the case of the channels that participate in the CCE pathway [Barritt, 1999]. Ca^{2+} readdition to control cells treated with vehicle alone resulted in no significant changes in Ca^{2+} levels (data not shown) thus precluding either CCE being activated by passive store depletion as a consequence of the absence of external calcium or a leaky state of the cells.

A wide variety of cells and tissues, including breast cancer cells [Flezar and Heisler, 1993; Enomoto et al., 1994], respond to extracellular nucleotides with an increase of $[Ca^{2+}]_i$ due to the stimulation of specific membrane purinergic receptors of the P2Y type [Ralevic and Burnstock, 1998] but relatively little is known about their exact physiological significance. As mentioned above, MCF-7 cells express $P2Y_2$ receptors that are activated by ATP [Flezar and Heisler, 1993]. The activation of PLC coupled to these receptors leads to phosphoinositide breakdown with the resultant formation of IP_3 and the mobilization of Ca^{2+} from endogenous stores. We next evaluated the ability of ATP (10 μM) to activate CCE in these cells by means of the above-mentioned Ca^{2+} free/Ca^{2+} back protocol. In other cell types, micromolar concentrations of ATP induce a rapid increase in $[Ca^{2+}]_i$ [De Souza et al., 1995; Wiebe et al., 1999; O'Neill et al., 2002]. Figure 1 also shows that stimulation of MCF-7 cells with 10 μM ATP induced a fast and transient increase in cytosolic Ca^{2+}, which returned to basal levels after 2–3 min of agonist exposure. Similarly to TG, Ca^{2+} readmission after the transient ATP-dependent Ca^{2+} elevation resulted in a fast increase in cytoplasmic Ca^{2+}, revealing the existence of an agonist-dependent CCE, activated as a consequence of receptor-induced depletion of intracellular Ca^{2+} stores. In both TG and ATP-induced CCE, once Ca^{2+} entry reached a maximum, it decayed to a steady-state level, with kinetics highly similar to that which followed the TG-induced transient calcium rise, thus suggesting that the plasma membrane Ca^{2+}-ATPase (PMCA) was the major calcium extruding system, as has been shown in other cell systems [Sedova and Blatter, 1999]. Depletion of Ca^{2+} stores with TG completely prevented the subsequent ATP-induced calcium mobilization (not shown) and conversely, cells pretreated with ATP were unable to respond to TG (Fig. 2). These data suggest that the two agents mobilize calcium from the same source.

ATP-induced Ca^{2+} mobilization and CCE were completely blocked using compound U-73122 (5 μM), an aminosteroid that has an inhibitory effect on different agonist-induced PLC-mediated biological activities in several cell types [Dubyak et al., 1988] (data not shown). The selectivity of U-73122 action was evidenced by using compound U-73343, an inactive analog of the former [Smith et al., 1990], which did not prevent the ATP stimulation of CCE in these cells. This provides evidence of an ATP-induced activation of the PLC pathway, which in turn mobilizes Ca^{2+} from the intracellular stores that triggers CCE through the plasma membrane.

When used in the micromolar range, Gd^{3+} acts as a selective blocker of CCE, becoming a useful pharmacological tool to study this mechanism [Broad et al., 1999; Jan et al., 1999; Moneer and Taylor, 2002]. Pretreatment of

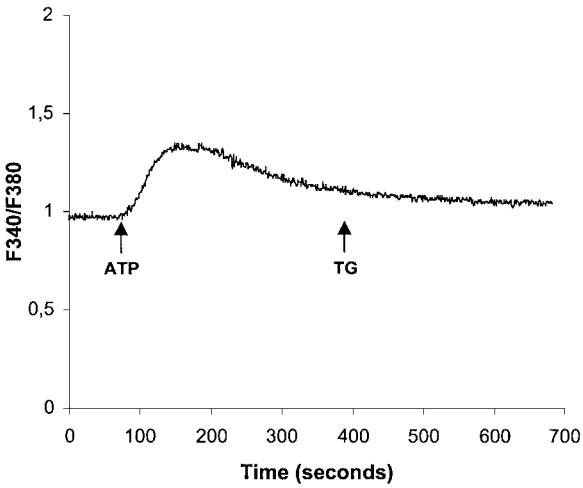

Fig. 2. Cells pretreated with ATP are unable to respond to thapsigargin. Fura-2 loaded cells were exposed to 10 μM ATP. The transient Ca^{2+} rise that followed treatment corresponds to the ATP-induced release of Ca^{2+} from inner stores. After cytosolic Ca^{2+} returned to basal, thapsigargin (TG; 500 nM) was added in order to test for Ca^{2+} remaining in the inner compartment.

MCF-7 cells with low micromolar concentrations of Gd^{3+} dose-dependently inhibited ATP-induced CCE without having any effects on the transient Ca^{2+} rise caused by the release of the cation from intracellular stores. At 1 and 5 μM Gd^{3+}, CCE was reduced by about 25 and 48%, respectively (not shown). Maximal inhibition (90%) was achieved at 10 μM (Fig. 3). The non-significant Ca^{2+} entry remaining at this Gd^{3+} concentration is a consequence of Ca^{2+} readdition to the medium. Compound 2-aminoethoxydiphenyl borate (2-APB), another CCE antagonist, at concentrations of 75 or 100 μM, also inhibited ATP stimulated capacitative Ca^{2+} entry when added either before (Fig. 4A) or after (Fig. 4B) the agonist. This observation is accounted for the fact that this compound has been shown to inhibit CCE by acting at the level of either the inositol 1,4,5-trisphosphate (IP_3) receptor [Maruyama et al., 1997] or the calcium entry pathway itself [Clapham et al., 2001; Iwasaki et al., 2001].

In various cell types, CCE is known to permeate Ba^{2+}, Sr^{2+}, and/or Mn^{2+} [Vazquez et al., 1998; Picotto et al., 1999], reflecting the fact that CCE is sometimes mediated by a heterogeneous population of channels with a

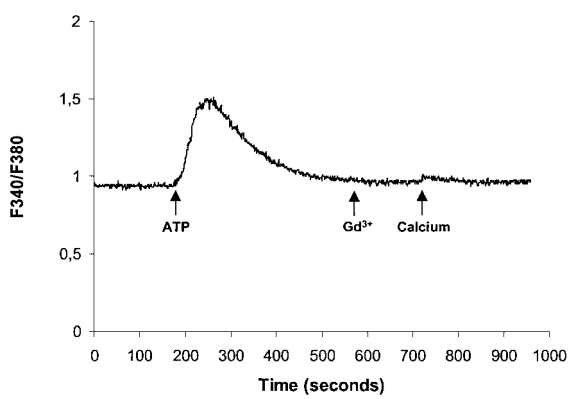

Fig. 3. Gd^{3+} blockade of ATP-induced CCE in MCF-7 cells. MCF-7 cells, incubated in Ca^{2+}-free medium, were treated with 10 μM ATP. Once the transient Ca^{2+} rise that followed store depletion returned to basal, Gd^{3+} (10 μM) was added and 2 min later Ca^{2+} (1.5 mM) was restored to the medium.

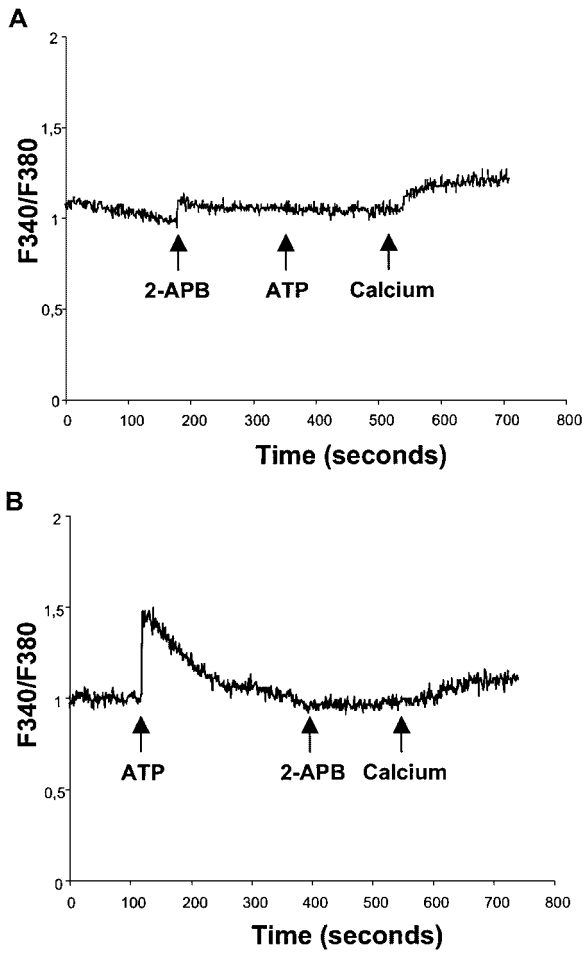

Fig. 4. Compound 2-APB prevents ATP-stimulated capacitative Ca^{2+} entry. Fura-2-loaded cells, incubated in Ca^{2+}-free medium, were treated as follows: (**A**) Cells were incubated with 2-APB (75 μM) for 3 min and then exposed to 10 μM ATP. Three min later Ca^{2+} (1.5 mM) was restored to the medium. (**B**) Cells were exposed to ATP and once the transient elevation in intracellular Ca^{2+} was over, they were treated with 2-APB for 3 min and then Ca^{2+} was restored to the extracellular medium (right arrow).

varying degree of divalent cation selectivity. Mn^{2+} and Ba^{2+} cannot be removed from the cytosol because they are not substrates for either SERCAs or PMCAs, offering a reliable way to monitor unidirectional entry of bivalent cations through the capacitative pathway [Palade et al., 1989; Byron and Taylor, 1995]. Using a Fura-2 fluorescence-quenching technique, we evaluated the ATP-induced permeability of CCE to Mn^{2+}. In MCF-7 cells, the CCE stimulated by ATP resulted in increased permeability to Mn^{2+} (Fig. 5A) and Mn^{2+} entry sensitivity to Gd^{3+} matched that of the Ca^{2+} entry pathway (Fig. 5B). These results suggest, at least on a pharmacological basis, that the same entry route mediated the influx of Ca^{2+} and Mn^{2+}. The ATP-stimulated CCE was also permeable to the calcium surrogates Ba^{2+} and Sr^{2+} in the same degree (Fig. 6). However, after reaching the peak of cation entry, Ca^{2+} and Sr^{2+} entry was followed by a decline phase as these cations can be handled by PMCAs [Broad et al., 1999; Moneer and Taylor, 2002] while Ba^{2+} remained at a plateau phase, as it cannot be pumped out of the cell [Palade et al., 1989]. As for Ca^{2+} readmission to cells not previously stimulated, when either Sr^{2+} or Ba^{2+} (1.5 mM) were restored to the extracellular medium, no significant changes in basal levels were observed (not shown).

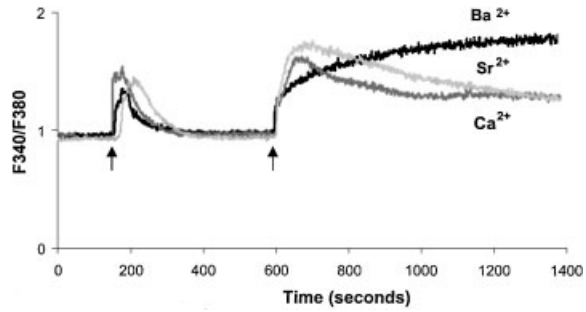

Fig. 6. Ca^{2+}, Sr^{2+}, and Ba^{2+} permeability of the ATP-induced CCE in MCF-7 cells. Fura-2 loaded cells were treated with 10 μM ATP (left arrow). Once the store depletion-dependent rise finished, Ca^{2+}, Sr^{2+} or Ba^{2+} (1.5 mM) were added to the extracellular medium (right arrow).

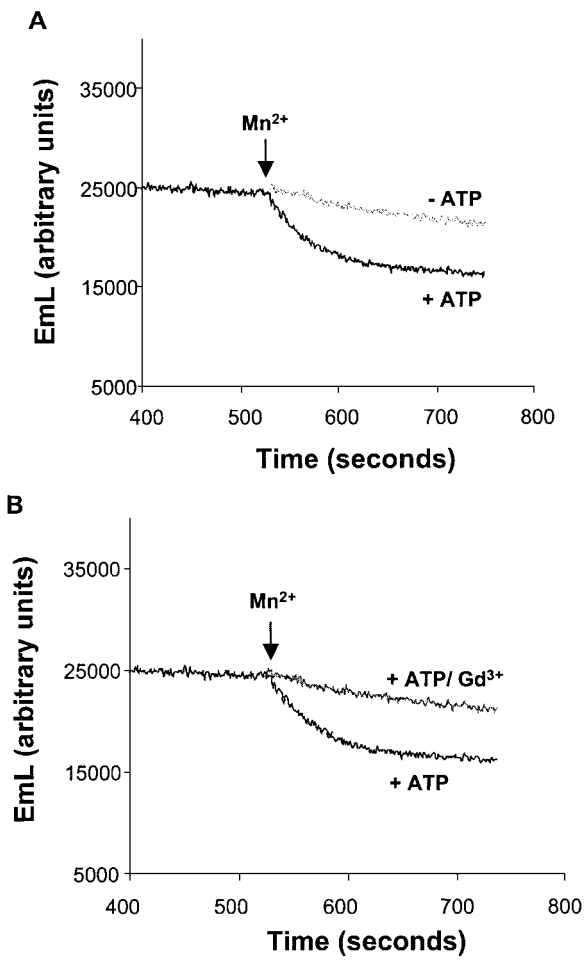

Fig. 5. Mn^{2+} permeability of the ATP-induced CCE in MCF-7 cells. Fura-2 loaded cells, incubated in Ca^{2+}-free medium, were treated with 10 μM ATP. Once the transient Ca^{2+} rise that followed store depletion occurred, cells were treated with: (**A**) Mn^{2+} (100 μM); (**B**) Gd^{3+} (10 μM), and 2 min later Mn^{2+} (100 μM) was added to the medium. Mn^{2+} entry was monitored by measuring the quenching of Fura-2 fluorescence at an excitation wavelength of 360 nm.

It is widely accepted that the major action of steroid hormones is through nuclear receptors and modification of the transcriptional activity of specific genes by the hormone-receptor complex [Hall et al., 2001]. However, rapid actions of estrogens have been reported in this and other cellular systems. Many experimental data suggest the existence of non-genomic effects that cannot be accounted for by the classic model of steroid action, but by receptors on the cell surface affecting second messenger signaling systems [Revelli et al., 1998; Hall et al., 2001; Levin, 2001, 2002]. The existence of a putative membrane estrogen receptor was first reported in endometrial cells and hepatocytes [Pietras and Szego, 1977; Pietras and Szego, 1980] and further studies revealed its presence in several cellular systems [Luconi et al., 1999; Razandi et al., 1999; Norfleet et al., 2000; Hall et al., 2001; Adams et al., 2002; Chambliss and Shaul, 2002]. By using immunocytochemistry, immunochemical and ligand binding analysis, there has been found evidence which suggest the

association of ER-like proteins to the plasma membrane fraction of MCF-7 cells [Monje et al., 2001]. Intracellular Ca^{2+} is involved in the rapid estrogen-membrane actions in other cell types [Picotto et al., 1999; Rubio-Gayosso et al., 2000; Perret et al., 2001; Sherman et al., 2002]. Here, we have evaluated the fast effects of 17β-estradiol on cytosolic Ca^{2+} and the possibility that estrogen modulates ATP-dependent CCE. As shown in Figure 7A, preincubation of Fura-2 loaded cells with 17β-estradiol (1 nM, 2 min) did not induce modifications in $[Ca^{2+}]_i$ by itself but completely blocked ATP-induced transient store depletion and CCE in MCF-7 cells. The rapid nature by which the steroid blocks ATP effects is compatible with the existence of a membrane-residing ER acting as the locus for this non-genomic action. To obtain evidence on whether an ER-like protein located at the plasma membrane might be involved in the rapid blockade of ATP-mediated effects, cells were treated with the monoclonal antibody Ab10 directed against the ligand binding region of human ER α. It was found that preincubation with the antibody abolished steroid hormone suppression of ATP-induced actions on MCF-7 cells (Fig. 7A). Cells incubated with control medium or antibody alone exhibited no changes in intracellular calcium concentration (not shown). Since it is highly unlikely that the antibody will enter the cells, these results support the idea that an ER-like protein residing at the cell surface could mediate 17β-estradiol inhibition of both ATP-dependent calcium release and entry. In agreement with this concept, Western blotting analysis using the same monoclonal antibody (Ab10) confirmed the existence of an ER-immunoreactive protein of 67 kDa in purified plasma membranes of MCF-7 cells but not in the endoplasmic reticulum fraction (Fig. 7B). Plasma membranes were not contaminated with endoplasmic reticulum as determined by its specific marker glucose-6-P phosphatase (not shown). The purity of the plasma membrane fraction was also established by immunoblot analysis revealing the absence of Lamin B, a nuclear specific protein, and the presence of Gαs, a plasma membrane-associated protein (Fig. 7C).

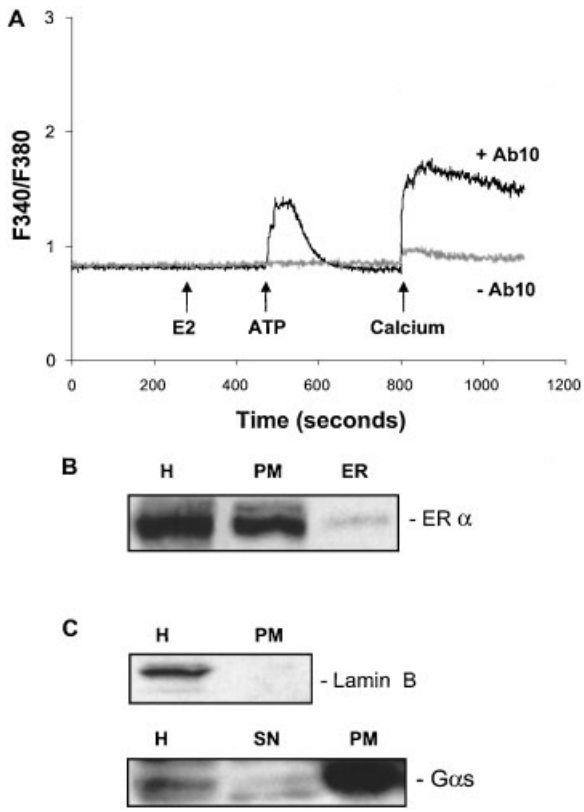

Fig. 7. 17β-estradiol effects on ATP-stimulated CCE. (**A**) Fura-2 loaded MCF-7 cells incubated in Ca^{2+}-free medium were incubated with 17β-estradiol (E2, 1 nM) for 2 min with (+Ab10) or without (−Ab10) a monoclonal antibody raised against the classical ERα-protein (Ab10, 1:400 dilution, final concentration, 10 min) and then treated with 10 μM ATP; 3 min later Ca^{2+} (1.5 mM) was added to the medium. (**B**) Western blot analysis of ER-like proteins residing at the plasma membrane of MCF-7 cells using the same monoclonal antibody as in panel A. (**C**) Distribution of the nuclear marker Lamin B and the plasma membrane-associated protein Gαs. H, total homogenate; PM, plasma membrane; ER, endoplasmic reticulum; SN, supernatant.

Moreover, 17β-estradiol (1 nM, 2 min) incubation of fura-2-loaded MCF-7 cells, prior to the incorporation of TG to the medium, did not influence the sesquiterpene-induced transient store depletion and CCE in these cells (Fig. 8). This suggests that estrogen blocks the CCE pathway activated by ATP acting up-stream of inner store Ca^{2+} release. The site of action of 17β-estradiol on the ATP-induced signaling pathway (e.g., $P2Y_2$ receptor, G-protein coupled PLC, IP_3 receptor) should be investigated. In agreement with these observations, there were recently reported non-genomic inhibitory effects of estrogens for several agonist-regulated processes initiated at the cell surface [Kim et al., 2000; Salom et al., 2001; Machado et al., 2002]. Of relevance in connection with this study, 17β-estradiol has been shown to inhibit ATP-induced catecholamine secretion in PC12 cells partly acting at the level of $P2Y_2$ receptors in a non-genomic manner [Kim et al., 2000].

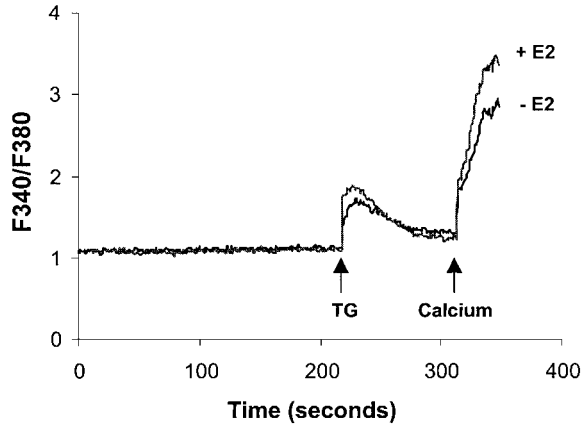

Fig. 8. 17β-estradiol does not alter thapsigargin-induced CCE. Fura-2 loaded MCF-7 cells incubated in Ca^{2+}-free medium were incubated in the absence and presence of 17β-estradiol (E2, 1 nM) for 2 min prior to the addition of 500 nM thapsigargin; once the transient Ca^{2+} rise was over, Ca^{2+} (1.5 mM) was added to the medium.

Our studies represent, to our knowledge, the first evidence to date on the existence of an ATP-driven, estrogen sensitive, CCE entry route in the human breast cancer epithelial cell line MCF-7. These observations may have biomedical implications, since extracellular ATP is known to inhibit the growth of various tumors [Spungin and Friedberg, 1993, and references therein]. In fact, it was recently shown that ATP inhibits oseophageal cancer cell growth through activation of the $P2Y_2$ receptors which leads to a rise in $[Ca^{2+}]_i$ [Maaser et al., 2002], of importance for modulation of cell differentiation, proliferation, and apoptosis [Sergeev and Rhoten, 1998]. Moreover, it has been reported that emptying the intracellular Ca^{2+} stores by 1,25-dihydroxyvitamin D_3 or its analog Ro-23-7553 is followed by the induction of apoptosis in MCF-7 cells [Vandewalle et al., 1995; Sergeev et al., 2000]. In view of the findings of the present study, one may suppose that 17β-estradiol, upon binding to the ER-like receptor in MCF-7 membranes, up-regulates cell proliferation by a non-genomic mechanism, which involves stimulation of the MAPK cascade [Razandi et al., 1999; Castoria et al., 1999; Lobenhofer et al., 2000], and also reversal of extracellular ATP inhibition of cell growth.

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REFERENCES

Adams MM, Fink SE, Sha RA, Janssen WG, Hayashi S, Milner TA, McEwen BS, Morrison JH. 2002. Estrogen and aging affect the subcellular distribution of estrogen receptor-alpha in the hippocampus of female rats. J Neurosci 22:3608–3614.

Aronica SM, Kraus WL, Katzenellenbogen BS. 1994. Estrogen action via the cAMP signaling pathway: Stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci USA 91:8517–8521.

Barritt GJ. 1999. Receptor-activated Ca^{2+} inflow in animal cells: A variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. Biochem J 337:153–169.

Berridge MJ. 1995. Inositol trisphosphate and calcium signalling. Nature 361:315–325.

Bers DM, Patton CW, Nuccitelli R. 1994. A practical guide to the preparation of Ca^{2+} buffers. Methods Cell Biol 40:3–29.

Broad LM, Cannon TR, Taylor CW. 1999. A non-capacitative pathway activated by arachidonic acid is the major Ca^{2+} entry mechanism in rat A7r5 smooth muscle cells stimulated with low concentrations of vasopressin. J Physiol 517:121–134.

Byron K, Taylor CW. 1995. Vasopressin stimulation of Ca^{2+} mobilization, two bivalent cation entry pathways and Ca^{2+} efflux in A7r5 rat smooth muscle cells. J Physiol 485:455–468.

Castoria G, Barone MV, Di Domenico M, Bilancio A, Ametrano D, Migliaccio A, Auricchio F. 1999. Non-transcriptional action of oestradiol and progestin triggers DNA synthesis. EMBO J 18:2500–2510.

Chambliss KL, Shaul PW. 2002. Rapid activation of endothelial NO synthease by estrogen: Evidence of steroid receptor fast-action complex (SRFC) in caveolae. Steroids 67:413–419.

Clapham DE. 1995. Calcium signaling. Cell 80:259–268.

Clapham DE, Runnels LW, Strüben C. 2001. The TRP ion channel family. Nature Rev Neurosci 2:387–396.

De Souza LR, Moore H, Raha S, Reed JK. 1995. Purine and pyrimidine nucleotides activate distinct signalling pathways in PC12 cells. J Neurosci Res 41:753–763.

Dubyak GR, Cowen DS, Meuller LM. 1988. Activation of inositol phospholipid breakdown in HL60 cells by P2-purinergic receptors for extracellular ATP. Evidence for mediation by both pertussis toxin-sensitive and pertussis toxin-insensitive mechanisms. J Biol Chem 263:18108–18117.

Enomoto K, Furuya K, Yamagishi S, Oka T, Maeno T. 1994. The increase in the intracellular Ca^{2+} concentration induced by mechanical stimulation is propagated via release of pyrophosphorylated nucleotides in mammary epithelial cells. Pflugers Arch 427:533–542.

Flezar S, Heisler J. 1993. P2-purinergic receptors in human breast tumor cells: Coupling of intracellular calcium signaling to anion secretion. J Pharmacol Exp Ther 265:1499–1510.

Graber R, Sumida C, Vallette G, Núñez E. 1993. Rapid and long-term effects of 17β-estradiol on PIP_2-phospholipase C coupling. Cell Signal 5:181–186.

Hall JM, Couse JF, Korach KS. 2001. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 276:36869–36872.

Harrison SM, Bers DM. 1987. The effect of temperature and ionic strength on the apparent Ca-affinity of EGTA and the analogous Ca-chelators BAPTA and dibromo-BAPTA. Biochim Biophys Acta 925:133–143.

Iwasaki H, Mori Y, Hara Y, Uchida K, Zhou H, Mikoshiba K. 2001. 2-aminoethoxydiphenyl borate (2-APB) inhibits capacitative calcium entry independently of the function of inositol 1,4,5-trisphosphate receptors. Receptors Channels 7:429–439.

Jan CR, Ho CM, Wu SN, Tseng CJ. 1999. Mechanism of rise and decay of thapsigargin-evoked calcium signals in MDCK cells. Life Sci 64:259–267.

Kim YS, MacDonald PN, Dedhar S, Hruska KA. 1996. Association of 1 alpha,25-dihydroxyvitamin D_3-occupied vitamin D receptors with cellular membrane acceptance sites. Endocrinology 137:3649–3658.

Kim YJ, Hur EM, Park TJ, Kim KT. 2000. Non-genomic inhibition of catecholamine secretion by 17β-estradiol in PC12 cells. J Neurochem 74:2490–2496.

Levin ER. 2001. Cell localization, physiology, and non-genomic actions of estrogen receptors. J Appl Physiol 91: 1860–1867.

Levin ER. 2002. Cellular functions of plasma membrane estrogen receptors. Steroids 67:471–475.

Lobenhofer EK, Huper G, Iglehart JD, Marks JR. 2000. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. Cell Growth Differ 11:99–110.

Luconi M, Muratori M, Forti G, Baldi E. 1999. Identification and characterization of a novel functional estrogen receptor on human sperm membrane that interferes with progesterone effects. J Clin Endocrinol Metab 84: 1670–1678.

Maaser K, Hopfner M, Kap H, Sutter AP, Barthel B, von Lampe B, Zeitz M, Sher H. 2002. Extracellular nucleotides inhibit growth of human oseophageal cancer cells via $P2Y_2$ receptors. Br J Cancer 86:636–644.

Machado JD, Alonso C, Morales A, Gomez FJ, Borges R. 2002. Nongenomic regulation of the kinetics of exocytosis by estrogens. J Pharmacol Exp Ther 301:631–637.

Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K. 1997. 2-APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P_3-induced Ca^{2+} release. J Biochem 122:498–505.

Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, Auricchio F. 1996. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 15:1292–1300.

Moneer Z, Taylor CW. 2002. Reciprocal regulation of capacitative and non-capacitative Ca^{2+} entry in A7r5 vascular smooth muscle cells: Only the latter operates during receptor activation. Biochem J 362:13–21.

Monje P, Zanello S, Holick M, Boland R. 2001. Differential cellular localization of estrogen receptor alpha in uterine and mammary cells. Mol Cell Endocrinol 181: 117–129.

Norfleet AM, Clarke CH, Gametchu B, Watson CS. 2000. Antibodies to the estrogen receptor-alpha modulate rapid prolactin release from rat pituitary tumor cells through plasma membrane estrogen receptors. FASEB J 14:157–165.

O'Neill AF, Hagar RE, Zipfel WR, Nathanson MH, Ehrlich BE. 2002. Regulation of the type III InsP(3) receptor by InsP(3) and calcium. Biochem Biophys Res Commun 294:719–725.

Palade P, Dettbarn C, Brunder D, Stein P, Hals G. 1989. Pharmacology of calcium release from sarcoplasmic reticulum. J Bioenerg Biomembr 21:295–320.

Perret S, Dockery P, Harvey BJ. 2001. 17beta-oestradiol stimulates capacitative Ca^{2+} entry in human endometrial cells. Mol Cell Endocrinol 176:77–84.

Picotto G, Vazquez G, Boland R. 1999. 17beta-oestradiol increases intracellular Ca^{2+} concentration in rat enterocytes. Potential role of phospholipase C-dependent store-operated Ca^{2+} influx. Biochem J 339:71–77.

Pietras RJ, Szego CM. 1977. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 265:69–72.

Pietras RJ, Szego CM. 1980. Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes. Biochem J 191:743–760.

Putney JW, Jr. 1997. Type 3 inositol 1,4,5-trisphosphate receptor and capacitative calcium entry. Cell Calcium 21:257–261.

Putney JW, Jr., Bird GS. 1993. The inositol phosphate-calcium signaling system in nonexcitable cells. Endocr Rev 14:610–631.

Ralevic V, Burnstock G. 1998. Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492.

Randriamampita C, Tsien RY. 1993. Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. Nature 364:809–814.

Razandi M, Pedram A, Greene GL, Levin ER. 1999. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: Studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. Mol Endocrinol 13:307–319.

Revelli A, Massobrio M, Tesarik J. 1998. Nongenomic actions of steroid hormones in reproductive tissues. Endocr Rev 19:3–17.

Rubio-Gayosso I, Sierra-Ramirez A, Garcia-Vazquez A, Martinez-Martinez A, Munoz-Garcia O, Morato T, Ceballos-Reyes G. 2000. 17 Beta-estradiol increases intracellular calcium concentration through a short-term and nongenomic mechanism in rat vascular endothelium in culture. J Cardiovasc Pharmacol 36:196–202.

Sadoshima J, Takahashi T, Jahn L, Izumo S. 1992. Roles of mechano-sensitive ion channels, cytoskeleton, and contractile activity in stretch-induced immediate-early gene expression and hypertrophy of cardiac myocytes. Proc Natl Acad Sci USA 89:9905–9909.

Salom JB, Burguete MC, Perez-Asensio FJ, Torregrosa G, Alborch E. 2001. Relaxant effects of 17β-estradiol in cerebral arteries through Ca^{2+} entry inhibition. J Cereb Blood Flow Metab 21:422–429.

Sedova M, Blatter LA. 1999. Dynamic regulation of $[Ca^{2+}]_i$ by plasma membrane $Ca(^{2+})$-ATPase and Na^+/Ca^{2+} exchange during capacitative Ca^{2+} entry in bovine vascular endothelial cells. Cell Calcium 25:333–343.

Sergeev IN, Rhoten WB. 1998. Regulation of intracellular calcium in human breast cancer cells. Endocrine 9:321–327.

Sergeev IN, Colby J, Norman AW. 2000. 1,25-dihydroxyvitamin D_3 triggers calcium-mediated apoptosis. In: Norman AW, Bouillion R, Thomasset M, editors. Vitamin D endocrine system: Structural, biological, genetic and clinical aspects. Riverside: University of California. p 399–402.

Sherman TS, Chambliss KL, Gibson LL, Pace MC, Mendelsohn ME, Pfister SL, Shaul PW. 2002. Estrogen acutely activates prostacyclin synthesis in ovine fetal pulmonary artery endothelium. Am J Respir Cell Mol Biol 26:610–616.

Smith RJ, Sam LM, Justen JM, Bundy GL, Bala GA, Bleasdale JE. 1990. Receptor-coupled signal transduction in human polymorphonuclear neutrophils: Effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. J Pharmacol Exp Ther 253: 688–697.

Spungin B, Friedberg I. 1993. Growth inhibition of breast cancer cells induced by exogenous ATP. J Cell Physiol 157:502–508.

Suzuki Y, Hruska KA, Reid I, Alvarez UM, Avioli LV. 1989. Characterization of phospholipase C activity of the plasma membrane and cytosol of an osteoblast-like cell line. Am J Med Sci 297:135–144.

Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+}stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc Natl Acad Sci USA 87:2466–2470.

Tsien RW, Tsien RY. 1990. Calcium channels, stores, and oscillations. Ann Rev Cell Biol 6:715–760.

Vandewalle B, Hornez L, Wattez N, Revillion F, Lefebvre J. 1995. Vitamin-D3 derivatives and breast-tumor cell growth: Effect on intracellular calcium and apoptosis. Int J Cancer 61:806–811.

Vazquez G, de Boland AR, Boland R. 1997. Stimulation of Ca^{2+} release-activated Ca^{2+} channels as a potential mechanism involved in non-genomic 1,25$(OH)_2$-vitamin D_3-induced Ca^{2+} entry in skeletal muscle cells. Biochem Biophys Res Commun 239:562–565.

Vazquez G, de Boland AR, Boland RL. 1998. 1alpha,25-dihydroxy-vitamin-D_3-induced store-operated Ca^{2+} influx in skeletal muscle cells. Modulation by phospholipase C, protein kinase C, and tyrosine kinases. J Biol Chem 273:33954–33960.

Wiebe SH, Sims SM, Dixon SJ. 1999. Calcium signalling via multiple P2 purinoceptor subtypes in rat osteoclasts. Cell Physiol Biochem 9:323–337.

Wiemann M, Busselberg D, Schirrmacher K, Bingmann D. 1998. A calcium release activated calcium influx in primary cultures of rat osteoblast-like cells. Calcif Tissue Int 63:154–159.

Wiemann M, Schirrmacher K, Busselberg D. 1999. Interference of lead with the calcium release activated calcium flux of osteoblast-like cells. Calcif Tissue Int 65:479–485.