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Capacitative Calcium Influx in Human Epithelial Breast Cancer and Non-Tumorigenic Cells Occurs Through Ca^{2+} Entry Pathways With Different Permeabilities to Divalent Cations

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Abstract The operation of capacitative Ca^{2+} entry (CCE) in human breast cancer (SKBR3) and non-tumorigenic (HBL100) cell lines was investigated as an alternative Ca^{2+} entry route in these cells. Ca^{2+} readdition after thapsigargin-induced store depletion showed activation of CCE in both cell lines. SKBR3 cells exhibited retarded store depletion and CCE decay kinetics compared to the non-tumorigenic HBL100 cells, suggesting alterations in Ca^{2+} homeostasis. CCE was also highly permeable to Mn^{2+} and to a lesser extent to Sr^{2+}, but not to Ba^{2+}. In HBL100 cells, CCE is contributed (30%) by a Ca^{2+}/Mn^{2+} permeable route insensitive to low (1 μM) Gd^{3+} and a $Ca^{2+}/Sr^{2+}/Mn^{2+}$ permeable non-selective pathway (70%) sensitive to 1 μM Gd^{3+}. In SKBR3 cells, the relative contribution to CCE of both routes was opposite to that in non-tumorigenic cells. J. Cell. Biochem. 88: 1265–1272, 2003.

Key words: capacitative Ca^{2+} entry; Ca^{2+} influx; Ca^{2+} regulation; breast cancer

Hormone-dependent regulation of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in non-excitable cells involves agonist-induced modulation of either Ca^{2+} release from inner stores by activation of the phospholipase C (PLC)/inositoltrisphosphate (IP_3) pathway, or stimulation of extracellular Ca^{2+} entry across Ca^{2+} channels in the plasma membrane, or both [Putney and Bird, 1993; Clapham, 1995]. Ca^{2+} sequestration into endogenous membrane stores, Ca^{2+} buffering by cytosolic binders and Ca^{2+} extrusion to the outside through both the Na^+–Ca^{2+} exchanger (NCX) and the Ca^{2+}-ATPase at the plasma membrane (PMCA) function in concert with the above mechanisms to determine $[Ca^{2+}]_i$. The effective cytosolic Ca^{2+} level and its fluctuations are intimately linked to diverse cellular processes such as proliferation and differentiation, secretion, cell metabolism, cytotoxicity and apoptosis, among others, all of them dependent on Ca^{2+}-signaling within the cell. Although several lines of evidence indicate that Ca^{2+} homeostasis may be altered in tumorigenic cells [Bergmann et al., 1994; Jain and Trump, 1997], information regarding intracellular Ca^{2+} regulation in cancer cells, particularly breast cancer cells, is scarce. Knowledge on the mechanisms of Ca^{2+} signaling in mammary gland cancer cells would be useful to elucidate the process of carcinogenesis as well as to contribute to the design of improved or new therapeutic strategies for this disease. In fact, many traditional breast cancer therapeutic agents are known to exert their influence through the Ca^{2+} signaling pathway [Bergmann et al., 1994; Sokolova et al., 1995; Jain and Trump, 1997]. Voltage-dependent Ca^{2+} channels (VDCCs), particularly those from the L-type, have been described in

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breast cancer cells, and partially account for the sustained Ca^{2+} influx phase frequently associated to the Ca^{2+} response of these cells to hormonal stimuli linked to activation of the PLC/IP_3 pathway [Patel and Schrey, 1992; Improta-Brears et al., 1999]. However, VDCCs may not represent the sole Ca^{2+} entry route in breast epithelial cells. In non-excitable cells the capacitative Ca^{2+} entry pathway (CCE or SOC, store-operated Ca^{2+} entry, will be unambiguously used within 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]. Recently, a main Ca^{2+} entry route has been described in the human BT-20 breast cancer cell line [Sergeev and Rhoten, 1998] which seems to involve non-selective voltage-independent Ca^{2+} channels but not sensitive to store depletion, and thus not responsible for mediation of capacitative Ca^{2+} entry. In the present work, we report for the first time the existence of a genuine store-operated, capacitative Ca^{2+} influx, in both non-tumorigenic (HBL100) and tumorigenic (SKBR3) human mammary gland epithelial cells lines which involves a heterogenous population of pharmacologically distinguishable non-selective cation channels which differentially contribute to total CCE.

MATERIALS AND METHODS

Chemicals

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

Cell Culture

HBL100 (non-tumorigenic) and SKBR3 (tumorigenic) human mammary gland epithelial established cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were cultured as monolayers onto glass coverslips at 37°C in RPMI medium containing 10% fetal bovine serum under humidified air (5.5% CO_2). Intracellular calcium measurements were made at confluence (2–4 days in culture).

Intracellular Calcium Measurements

Intracellular Ca^{2+} changes were monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2 as previously described [Baldi et al., 2002]. 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 seroalbumin (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. 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 ratio of Fura-2 fluorescence due to excitation at 340 nm to that due to excitation at 380 nm (340/380). Ca^{2+}-free extracellular medium means free Ca^{2+} concentration 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. Functional isolation of CCE in cells co-expressing VDCCs requires

suppression of the large Ca^{2+} influx that normally occurs through these channels [Vazquez et al., 1998; Broad et al., 1999]. As in previous studies [Vazquez et al., 1998; Baldi et al., 2002], we achieved this by incorporating the specific VDCC blockers nifedipine (5 μM) and verapamil (5 μM) in all incubating media to effectively block VDCC-mediated Ca^{2+} influx.

RESULTS

The capacitative Ca^{2+} entry (CCE) pathway in both HBL100 (non-tumorigenic) and SKBR3 (tumorigenic) human mammary gland epithelial cells was evaluated by using a Ca^{2+} re-addition protocol after depleting endogenous Ca^{2+} stores with the specific sarco/endoplasmic Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin.

In both HBL100 and SKBR3 cells, irreversible and specific inhibition of SERCAs with thapsigargin (500 nM) induced a pronounced and transient increase in $[Ca^{2+}]_i$ consequence of store-depletion through the leak channel [Pozzan et al., 1994], decaying to basal levels within 4–5 min (Fig. 1A). At this point, re-addition of Ca^{2+} to the extracellular millieu resulted in a rapid and prominent elevation of cytosolic Ca^{2+} (2.5/3-fold over basal values) reflecting activation of a capacitative, store depletion-operated pathway [Putney, 1986, 1990; Putney and Bird, 1993]. Ca^{2+} re-admission to cells not previously exposed to thapsigargin but maintained for at least 5 min in Ca^{2+}-free medium only caused a negligible increase in cytosolic Ca^{2+} (Fig. 1B), ruling out the possibility that the Ca^{2+} entry observed in Figure 1A reflected a leaky state of the cells. In HBL100 cells, once the maximum level of thapsigargin-induced CCE was achieved, the Ca^{2+} overshoot decayed with a kinetics highly comparable to the declining phase of the thapsigargin-induced Ca^{2+} transient (Fig. 1A), suggesting that the plasma membrane Ca^{2+} ATPase (PMCA) was the main mechanism of Ca^{2+} extrusion, as established in other cell systems [Sedova and Blatter, 1999; Snitsarev and Taylor, 1999 and see below]. Interestingly, the declining phase of CCE exhibited a delayed kinetics in SKBR3 cells, compared to that in HBL100 cells.

As shown in Figure 2A, inhibition of the plasma membrane Na^+/Ca^{2+} exchanger (NCX) by omitting Na^+ in the extracellular medium (replaced in equimolar amounts by Li^+) caused a modest, almost insignificant retardation in

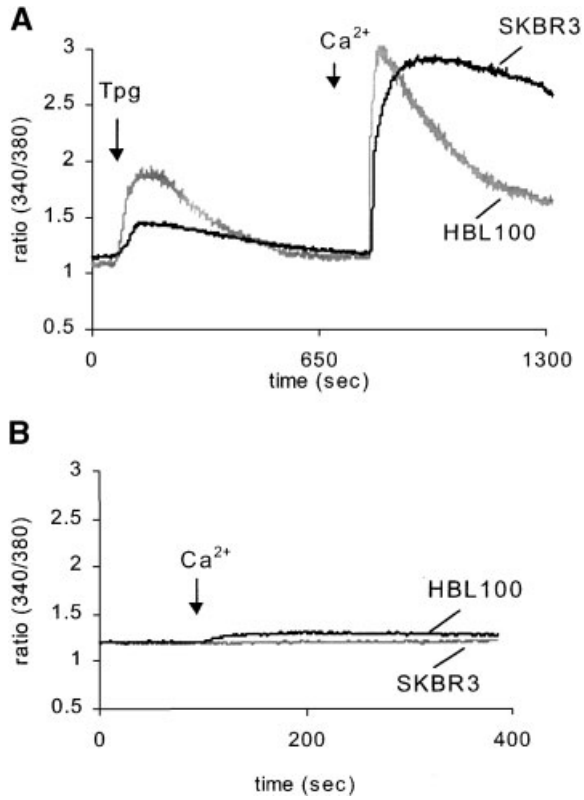

Fig. 1. Basal and thapsigargin-induced capacitative Ca^{2+} entry (CCE) in HBL100 and SKBR3 breast cells. Non-tumorigenic (HBL100) or tumorigenic (SKBR3) breast cells loaded with Fura-2 were incubated in Ca^{2+}-free medium containing 5 μM nifedipine and 5 μM verapamil, in order to block Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs). **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 preactivated pathway. **B**: Cells were incubated for at least 5 min in Ca^{2+}-free medium without pharmacological perturbation of the stores prior to Ca^{2+} re-addition (1.5 mM). Shown are time traces representative from at least five independent experiments, each performed onto no less than 50 cells.

the recovery from the Ca^{2+} overshoot in HBL100 cells, indicating that this decline of cytosolic Ca^{2+} was not mediated, or it was to a very limited extent, by NCX. In tumorigenic SKBR3 cells, NCX inhibition almost suppressed the already slow $[Ca^{2+}]_i$ decay that followed the CCE overshoot (Fig. 2B). The rate of decay of the transient increase in cytosolic Ca^{2+} caused by thapsigargin was not affected by extracellular Na^+ depletion in either cell type (not shown). Interestingly, the peak Ca^{2+} transient consequence of the depletion phase of the thapsigargin response was about 1.4-fold higher in HBL100 than in SKBR3 cells (Fig. 1A). Also, the kinetics of recovery from this peak Ca^{2+}

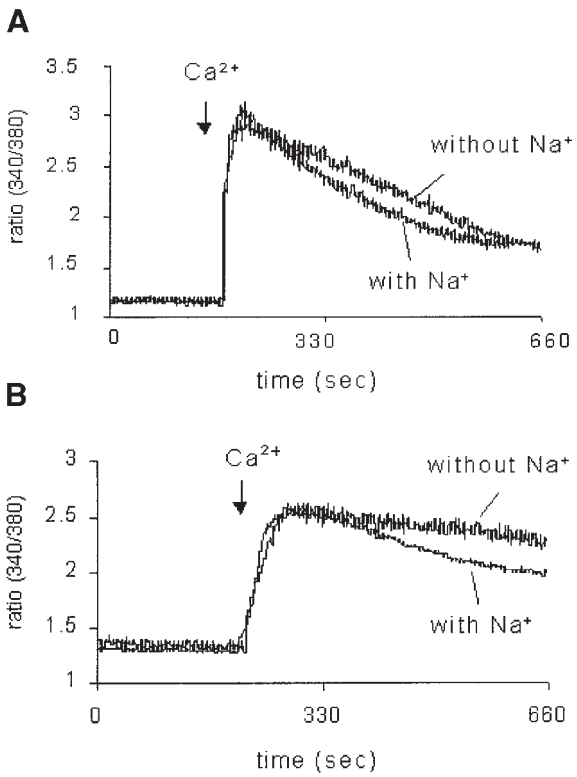

Fig. 2. Relative contribution of the Na^+–Ca^{2+} exchanger to the recovery phase of CCE in HBL100 and SKBR3 cells. **A**: Non-tumorigenic (HBL100) or (**B**) tumorigenic (SKBR3) breast cells loaded with Fura-2 were incubated in Ca^{2+}-free medium and inner stores were depleted with 500 nM thapsigargin (Tpg; see A in Fig. 1). After cytosolic Ca^{2+} returned to basal levels (for simplification, time-trace curves are shown from hereon), Ca^{2+} (1.5 mM) was re-added to the medium. As indicated, Ca^{2+} extrusion at the recovery phase of CCE was monitored with Na^+ (138 mM) or without Na^+ (replaced by equimolar amounts of Li^+) in the extracellular medium to inhibit the Na^+/Ca^{2+}-exchanger. Shown are time traces representative from at least three independent experiments, each performed onto no less than 50 cells.

transient was significantly slowed (2.0–2.5-fold) in SKBR3 cells but not affected by extracellular Na^+ depletion, ruling out a significant contribution from NCX.

In various cell types the CCE pathway permeates Ba^{2+}, Mn^{2+}, and/or Sr^{2+}; thus, these cations acting as Ca^{2+} surrogates, can be used as indicators of cation entry by monitoring their effects on cytosolic Fura-2 fluorescence. Both Ba^{2+} and Sr^{2+} when interacting with Fura-2 produce an excitation spectrum and maximal brightness highly similar to Ca^{2+}, whereas Mn^{2+} quenches Fura-2 fluorescence [Kwan and Putney, 1990]. As neither Ba^{2+} nor Mn^{2+} can be removed from cytosol because they are not substrates for SERCAs or PMCAs [Palade et al., 1989; Byron and Taylor, 1995], these cations provide a reliable way to monitor unidirectional entry of bivalent cations through CCE channels. In both HBL100 and SKBR3 cells, CCE induced by thapsigargin-dependent store-depletion was not permeable to Ba^{2+} (not shown). However, the thapsigargin-induced CCE in HBL100 cells resulted to be highly permeable to Mn^{2+} (Fig. 3), what constitutes a hallmark in almost all of the cell types where store-depletion operated cation influx pathways have been described [Putney and Bird, 1993; Fasolato et al., 1994; Berridge, 1995]. Similar results were obtained regarding Mn^{2+} permeability of the CCE pathway in SKBR3 cells (not shown).

In both cell types, CCE was also permeable to the Ca^{2+} surrogate Sr^{2+}, although the magnitude of fluorescence increase reflecting Sr^{2+} influx following store depletion was significantly lower than that when Ca^{2+} was re-added instead (~35% vs. 170% over basal, for Sr^{2+} influx vs. Ca^{2+} entry, respectively; Fig. 4). The Sr^{2+} influx phase was followed by a rapid decline to pre-stimulation levels, in line with the fact that this cation can be handled by PMCAs [Palade et al., 1989; Broad et al., 1999].

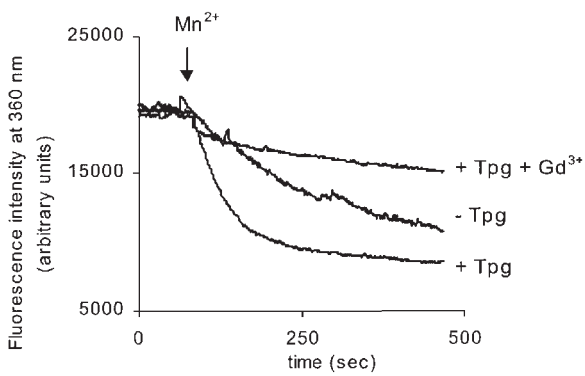

Fig. 3. Mn^{2+} permeability of thapsigargin-induced CCE Ca^{2+} entry in HBL100 cells. Non-tumorigenic HBL100 breast cells loaded with Fura-2 were incubated in Ca^{2+}-free medium and then treated with either vehicle (basal) or 500 nM thapsigargin (Tpg). Once the transient Ca^{2+} rise that followed store depletion by the Ca^{2+} mobilizing agent occurred, Mn^{2+} (100 μM) was added to the medium. Mn^{2+} entry was monitored as the quenching of Fura-2 fluorescence measured at an excitation wavelength of 360 nm (see Materials and Methods). When Gd^{3+} was used (indicated), it was added at a concentration of 1 μM after the thapsigargin-dependent transient Ca^{2+} rise took place, and 2 min later Mn^{2+} (100 μM) was added to the medium. Shown are time traces (fluorescence intensity at 360 nm; arbitrary units) representative from at least three independent experiments, each performed onto no less than 50 cells.

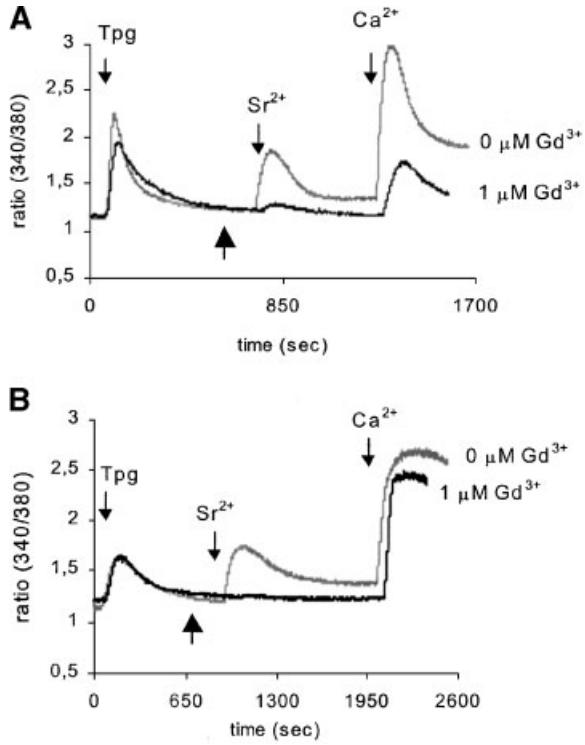

Fig. 4. Sr^{2+} permeability of thapsigargin-induced CCE in HBL100 and SKBR3 cells. **A**: Non-tumorigenic (HBL100) or (**B**) tumorigenic (SKBR3) breast cells loaded with Fura-2 were incubated in Ca^{2+}-free medium and then treated with 500 nM thapsigargin (Tpg). Once the store depletion-dependent transient Ca^{2+} rise occurred, when used, Gd^{3+} (1 μM) was added (short arrowhead), and 2 min later Sr^{2+} (1.5 mM) was added. After full recovery from the cytosolic Sr^{2+} rise that followed Sr^{2+} entry (see text), Ca^{2+} (1.5 mM) was re-added to the incubation medium. Gray curves show the behavior of Sr^{2+} and Ca^{2+} permeability in the absence of Gd^{3+}. Time traces representative from at least three independent experiments are given, each performed onto no less than 50 cells.

The kinetics of recovery from the peak Sr^{2+} entry was slower in SKBR3 (1.0–1.5-fold) than in HBL100 cells (Fig. 4B). Store-depletion activated Sr^{2+} entry did not affect the influx of Ca^{2+} by subsequent addition of this cation.

When used in the low micromolar range, Gd^{3+} behaves as a selective blocker of CCE, being thus particularly useful in cells in which various Ca^{2+} entry pathways co-exist [Broad et al., 1999]. Pre-treatment of breast cancer and non-tumorigenic cells with 1 μM Gd^{3+} inhibited thapsigargin-stimulated Sr^{2+} and Ca^{2+} entry (Fig. 4). However, sensitivity of these two cation entry pathways to the lanthanide blocker was clearly different. 1 μM Gd^{3+} completely prevented capacitative Sr^{2+} influx in both cell lines, whereas subsequent Ca^{2+} re-addition was reduced in 70% and 25% in HBL100 and SKBR3 cells, respectively, compared to non-Gd^{3+} treated cells. Concentrations of Gd^{3+} equal to or higher than 2.5 μM were required to completely block Ca^{2+} entry in both cell lines (not shown). Interestingly, Mn^{2+} entry sensitivity to Gd^{3+} in both HBL100 (Fig. 3) and SKBR3 (not shown) cells closely matched that of the Ca^{2+} entry pathway. It should be noted here that for the set of experiments shown in Figure 3, we were expecting that 1 μM Gd^{3+} would reduce store-operated Mn^{2+} entry at a level close or equal to the basal (−Tpg in Fig. 3); however, note that basal Mn^{2+} entry is actually higher than store-operated Mn^{2+} entry in the presence of the lanthanide blocker. The simplest explanation for this apparent discrepancy is that a population of low-Gd^{3+} sensitive non-store operated channels with low constitutive activity allows for Mn^{2+} entry and its consequent accumulation into the cytosol, as this cation is not subject to neither the extrusion nor the buffering systems that do affect Ca^{2+} entering the cell, thus becoming detectable by changes in Fura-2 fluorescence (the reason why this non-CCE low constitutive channel activity is not noticeable when Ca^{2+} is re-added to the bath; see for instance Fig. 1B). In fact, when assaying Ba^{2+} permeability of the CCE pathway (Ba^{2+} as Mn^{2+}, is not subject to neither extrusion nor buffering once it enters cytosol) in both HBL100 and SKBR3 cells, addition of Ba^{2+} (1.5 mM) to cells maintained for at least 5 min in nominally Ca^{2+} free medium resulted in appearance of a low but significant (~20% increase over basal signal) Ba^{2+} entry, no further regulated by store-depletion, suggestive again of the existence of a constitutively active non-CCE cation entry pathway. The remaining Mn^{2+} entry observed in Gd^{3+}-treated cells, then most likely reflects the component of the CCE route which is non-sensitive to 1 μM Gd^{3+} (see Fig. 4A). Thus, in HBL100 cells, the Sr^{2+} permeable CCE pathway strongly resembled the Ca^{2+}/Mn^{2+} permeable component of the total CCE that was sensitive to 1 μM Gd^{3+}. Altogether these results suggest that in HBL100 cells the capacitative Ca^{2+} entry pathway observed under pharmacological store depletion is contributed by at least two entry routes: a Ca^{2+}/Mn^{2+} permeable pathway insensitive to 1 μM Gd^{3+}, accounting for about 30% of the total CCE, and a quantitatively more important $Ca^{2+}/Sr^{2+}/Mn^{2+}$ permeable non-selective cation entry pathway fully inhibited

by very low (1 μM) micromolar Gd^{3+}. Although the breast cancer SKBR3 cell line exhibits a CCE route mediated by essentially the same cation entry pathways as those characterized in HBL100 cells, the relative contribution of each of these two pathways to total CCE was the oppossite to that observed for the non-tumorigenic cells.

DISCUSSION

In the present work we report for the first time the existence of a capacitative Ca^{2+} entry (CCE) pathway in both non-tumorigenic and tumorigenic human mammary gland epithelial cell lines. As in previous investigations [Vazquez et al., 1998; Baldi et al., 2002], we used a protocol that consists in restoring Ca^{2+} to the extracellular medium after depletion of endogenous Ca^{2+} stores with the specific SERCA inhibitor thapsigargin. This Ca^{2+} re-addition (Ca^{2+} free/Ca^{2+} back) maneuver has been extensively shown to be a convenient way to fluorimetrically monitor changes in Ca^{2+} influx through the CCE pathway [Putney and Bird, 1993; Birnbaumer et al., 1996; and references therein]. However, in cells known to express VDCCs, Ca^{2+} influx through these channels may occur when the cation is added back to the medium. As VDCCs have been described in breast cancer cells [Patel and Schrey, 1992; Improta-Brears et al., 1999], we applied the above mentioned Ca^{2+} re-addition scheme but incorporating highly specific VDCC blockers in all incubating media. By doing so, we were able to selectively isolate CCE function, as previously done in both skeletal muscle and osteoblastic cells [Vazquez et al., 1998; Baldi et al., 2002] and aortic smooth muscle cells [Broad et al., 1999].

A number of interesting observations have been made in the course of the present experiments. We found that in both HBL100 and SKBR3 cells, irreversible inhibition of SERCAs effectively results in store-depletion through the leak channel [Pozzan et al., 1994], and that Ca^{2+} restoration to the extracellular millieu results in rapid and robust elevation of cytosolic Ca^{2+} as a consequence of activation of a capacitative pathway. This represents the first genuine description to date revealing the existence of store-operated calcium channels in human breast epithelial cells as an alternative cation entry route besides the voltage-dependent pathway. In fact, VDCCs from the L-type class (nifedipine- and verapamil-sensitive) seem to be involved in the sustained Ca^{2+} influx phase of hormone-stimulated breast cancer cells [Patel and Schrey, 1992; Improta-Brears et al., 1999]. Far from being conflicting, the co-existence of these two differentially operated pathways provides a very interesting scenario for concerted regulation of calcium entry into these cells under physiological conditions, as it has been shown in other non-excitable cell systems that calcium influx through store-operated channels activated by external stimuli that mobilize calcium through the PLC/IP_3 cascade, induces a partial and transient membrane depolarization that next drives activation of the VDCC pathway responsible for a more sustained and robust calcium entry into the cell [Liu and Gylfe, 1997; Miura et al., 1997; Fomina and Nowycky, 1999].

When the kinetics of decay of the calcium overshoot that follows CCE activation was analyzed, we found that the plasma membrane Ca^{2+} ATPase (PMCA) seems to be the main mechanism of Ca^{2+} extrusion, whereas the Na^+/Ca^{2+} exchanger (NCX) has a very limited contribution (Figs. 1 and 2). This is in agreement with observations made in other non-excitable cell systems, such as vascular endothelial cells, where a role for PMCA as a major, if not exclusive, extrusion system in the recovery phase of CCE has been shown [Sedova and Blatter, 1999; Snitsarev and Taylor, 1999]. Interestingly, the tumorigenic SKBR3 cells exhibited a marked slower recovery from peak CCE when compared with their non-tumorigenic counterparts HBL100 cells; in some experiments, it reached the extreme situation of no recovery at all, i.e., a sustained, long lasting CCE. Although it is possible that sustained CCE in SKBR3 cells could be due to lack of Ca^{2+}-dependent inactivation of CCE channels, rather than (or combined to) diminished extrusion capacity of the PMCA, up to the present Ca^{2+}-dependent inactivation has been clearly proven for the highly Ca^{2+} selective CRAC channels (extensively discussed by Berridge, [1995]); although we cannot exclude expression of CRAC channels in breast cells, we do not expect them to represent the predominant Ca^{2+} entry route in these cells [as is the case for instance in hematopoietic cells, see Parekh and Penner, 1997] as our data indicate that CCE in both HBL100 and SKBR3 cells is mainly a non-selective cation entry

pathway. Moreover, NCX inhibition in SKBR3 cells resulted in a small decrease of the already slow decay that followed the CCE overshoot (Fig. 2B) indicating that, as is the case in HBL100 cells, NCX has a poor participation in the recovery from the Ca^{2+} overshoot, and reinforcing the notion that most likely a diminished PMCA activity is responsible for the slowered recovery from peak CCE.

Another important observation is that, although no significant differences were observed in either the initial rate of CCE or its amplitude at steady state when comparing HBL100 vs. SKBR3 cells, the peak Ca^{2+} transient that follows thapsigargin-induced store depletion (Fig. 1A) was significantly and consistently higher in HBL100 than in SKBR3 cells. We consider this unlikely to be due to incomplete depletion of the inner stores, as the magnitude of Ca^{2+} entry through the CCE pathway is known to be related to the degree of store depletion [Vazquez et al., 1998] and, as mentioned above, no differences were observed in either initial rate or amplitude of CCE. Since the kinetics of Ca^{2+} release following SERCA inhibition is highly similar in both cell types, the observed difference most likely reflects an intrinsic feature of the thapsigargin-sensitive pool in SKBR3 cells (such as Ca^{2+} size of the thapsigargin-sensitive store), rather than alterations in the SERCA itself. We speculate that alterations in the PMCA could account for such difference. The experiments showing CCE permeability to the Ca^{2+} surrogate Sr^{2+} (Fig. 4) are in line with this interpretation. The Sr^{2+} influx phase was followed by a rapid decline to prestimulation levels, in line with the fact that this cation can be handled by PMCAs [Palade et al., 1989; Broad et al., 1999]. Again, the kinetics of recovery from the peak Sr^{2+} entry was slower in SKBR3 compared to that in HBL100 cells; as the NCX does not handle Sr^{2+} [Matsuda et al., 1997] and no feedback nor buffering mechanisms (SERCA has been irreversibly blocked by thapsigargin) are involved in the kinetics of $[Sr^{2+}]_i$ decay, time-course of cytosolic Sr^{2+} levels represents genuine PMCA extrusion activity thus suggesting again that alterations in the PMCA may account for such altered Sr^{2+} extrusion kinetics. Whether or not the observed differences are somehow related to the tumorigenic nature of the SKBR3 cell line requires further molecular and biochemical experimental evaluation.

Finally, based on the fact that in different cell types CCE permeates the Ca^{2+} surrogates Ba^{2+}, Mn^{2+} and/or Sr^{2+} [Putney and Bird, 1993], we characterized the CCE pathway in both HBL100 and SKBR3 cells in terms of selectivity to divalent cations. Also, we performed these experiments in the presence or absence of the lanthanide Gd^{3+} which within the low micromolar range behaves as a selective blocker of CCE [Broad et al., 1999], in order to evaluate the homogeneity of the channel population involved in such a pathway in terms of their sensitivity to Gd^{3+}.

We found that in both HBL100 and SKBR3 cells the store-operated cation entry route was not permeable to Ba^{2+} but highly permeable to Mn^{2+} and Sr^{2+}, in line with observations made in other cell types where store-depletion operated channels have been described [Putney and Bird, 1993; Fasolato et al., 1994]. Based on the sensitivity of divalent cation permeation through the CCE pathway to Gd^{3+}, we found that, under pharmacological store depletion, this route is contributed, at least, by a Ca^{2+}/Mn^{2+} permeable pathway not sensitive to very low micromolar (1 μM) Gd^{3+}, and a quantitatively more significant $Ca^{2+}/Sr^{2+}/Mn^{2+}$ permeable non-selective cation entry pathway highly sensitive to very low (1 μM) micromolar Gd^{3+}. Although in both the breast cancer SKBR3 cell line and its non-tumorigenic counterpart CCE involves operation of essentially the same cation channel populations in terms of their cation selectivity and sensitivity to the lanthanide Gd^{3+}, the relative contribution of these two pathways to total CCE was significantly different in each of these cell lines. Once again, whether or not this differential contribution is due to differences in channel expression and/or regulation linked somehow to the tumorigenic vs. non-tumorigenic nature of these two cell types is by now speculative and demands further molecular and biochemical experimental evaluation.

In summary, the present studies describe the operation of a capacitative Ca^{2+} entry in both non-tumorigenic and tumorigenic human mammary gland epithelial cell lines involving a heterogenous store-operated non-selective cation influx pathway. As stated above, we propose that under physiological conditions this Ca^{2+} entry route may represent an alternative Ca^{2+} entry path through which extracellular stimuli can influence Ca^{2+} homeostasis in these cells.

In this context, the present findings set the basis to evaluate how calcium mobilizing hormones could regulate the CCE pathway in either breast cancer and non-tumorigenic epithelial cells, as part of their general mechanism of modulation of cellular function, proliferation, and differentiation of these cells.

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