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Heregulinβ activates store-operated Ca^{2+} channels through c-erbB2 receptor level-dependent pathway in human breast cancer cells

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Abstract

The heregulinβ (HRGβ) is a ligand to activate c-erbB2/c-erbB3 interaction and can subsequently increases cytosolic $[Ca^{2+}]_i$. In the two human breast cancer cell lines, MCF-7 shows a low c-erbB2 expression level, whereas SK-BR-3 overexpress c-erbB2 receptor. In this article, we have found that in MCF-7, HRGβ induced Ca^{2+} release from the endoplasmic reticulums (ER) and subsequently activated Ca^{2+} entry via store-operated Ca^{2+} channel (SOC). However, in SK-BR-3, HRGβ failed to induce Ca^{2+} release and Ca^{2+}entry. RNA interference to decrease c-erbB2 level in SK-BR-3 resulted in reactivation of HRGβ-evoked Ca^{2+} release and Ca^{2+} entry via SOC, which was similar to that of MCF-7. In addition, in the absence of HRGβ, a constitutive activation of SOC was observed in SK-BR-3 rather than in MCF-7 and c-erbB2-siRNA treated SK-BR-3. Compared to the cells with low c-erbB2 level, c-erbB2 might tend to interact with c-erbB3 in the resting state in the cells with high c-erbB2 level, which resulted in different $[Ca^{2+}]_i$ responses to HRGβ. In SK-BR-3, the Ca^{2+} mobilization in the presence or in the absence of HRGβ was completely blocked by PLC inhibitor U73122. In summary, our results indicate that HRGβ-induced SOC was regulated by c-erbB2 level and dependent on activation of PLC in human breast cancer cells.

Keywords: HRGβ; Store-operated Ca^{2+} channel (SOC); C-erbB2; PLC; Human breast cancer cells

Cytosolic Ca^{2+} signals represent a convergent point of many signal transduction pathways and modulate a diverse array of cellular activities ranging from short-term responses such as contraction and secretion, to longer-term control of transcription, cell division, and cell death. In non-excitable cells, intracellular calcium mobilization generally involves internal Ca^{2+} release from endoplasmic reticulums (ER) and following Ca^{2+} entry across the plasma membrane [1,2]. The stimulation of tyrosine kinase receptor by many hormones leads to stimulation of receptor tyrosine kinase-PLC-γ pathways to produce inositol 1,4,5-trisphosphate (IP_3). IP_3 couples to the IP_3 receptor (IP_3R)[3] located on ER and induces Ca^{2+} release [3,4]. The subsequent Ca^{2+} entry in this process is generally known as store-operated or capacitative Ca^{2+} entry through a specific type of Ca^{2+} channel termed store-operated Ca^{2+} channel (SOC) [1,2,5]. Previous studies have demonstrated that epidermal growth factor (EGF) induces Ca^{2+} signals via different mechanisms in different cell lines. Zhang et al. found

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[3] *Abbreviations used:* HRGβ, heregulinβ; SOC, store-operated Ca^{2+} channel; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; ER, endoplasmic reticulum; EGF, epidermal growth factor; EGFR, EGF receptor; TRP channels, transient receptor potential channels; RNAi, RNA interference; 2-APB, 2-aminoethoxydiphenyl borate; La^{3+}, lanthanide; U73122, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl) amino]hexyl]-1H-pyrrole-2,5-dione; Tg, thapsigargin; Fura-2/AM, Fura-2 acetoxymethyl ester; PVDF, polyvinylidene difluoride; TBST, tris-buffered saline with 0.1% Tween 20.

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that in human salivary cells, EGF stimulated Ca^{2+} influx by alternative mechanisms distinct from Ca^{2+} entry via SOC [6] and another laboratory provided evidence that EGF-activated SOC via a PLC-dependent but IP_3R independent pathway in human glomerular mesangial cells [7].

The erbB/epidermal growth factor receptor (EGFR) family consists of four proteins: EGFR (c-erbB1), c-erbB2, c-erbB3 and c-erbB4, which are involved in signal transduction pathways that regulate cell growth and differentiation. Overexpression or amplification of epidermal growth factor receptor is associated with malignancy and a poor prognosis in breast cancer. In the four receptors, c-erbB2 is overexpressed in about 30% of human breast cancer patients [8–11]. Heregulinβ (HRGβ) is a member of EGF-like ligands to lead to erbB-receptor interaction [11]. In the various heterodimers and homodimers, the c-erbB2/c-erbB3 dimer constitutes a highest affinity co-receptor for HRGβ [12–16], which results in activation of PLC signal pathway and subsequent mobilization of $[Ca^{2+}]_i$ [3,4,17,18]. EGF-induced rise in $[Ca^{2+}]_i$ via activation of EGFR is derived from the activation of both release from ER (triggered by IP_3) and Ca^{2+} influx via SOC located on plasma membrane [19–21]. However, whether Ca^{2+} influx stimulated by HRGβ is through SOC and the correlation between the c-erbB2 receptor level and signal transduction pathway modulated by cellular $[Ca^{2+}]_i$ are still unknown.

Here, we investigated the effects of c-erbB2 receptor at high and low levels on Ca^{2+} mobilization. Our study focused on MCF-7 and SK-BR-3 human breast cancer cell lines, representing models with different c-erbB2 expression level: MCF-7 shows a low c-erbB2 level, whereas SK-BR-3 is observed to overexpress c-erbB2 [22,23]. In the present study, we provided evidences that in the cells with low c-erbB2 level (MCF-7 and c-erbB2-siRNA treated SK-BR-3), HRGβ-evoked intracellular Ca^{2+} mobilization involved two phases (*i.e.*, an initial rapid rise followed by a sustained plateau). In SK-BR-3 cells with high level of c-erbB2 receptor, HRGβ failed to activate further increase of intracellular $[Ca^{2+}]_i$ although constitutive activation of SOC was measured without HRGβ addition. In addition, Ca^{2+} mobilization in the cells either with low c-erbB2 level or with high c-erbB2 level was via a PLC-dependent mechanism. Thus, c-erbB2 receptor level has an essential role in regulation of PLC-dependent Ca^{2+} mobilization pathway in human breast cancer cells.

Materials and methods

Materials

Heregulinβ (HRGβ), 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), Fura-2 acetoxymethyl ester (Fura-2/AM) and thapsigargin (Tg) were obtained from Sigma. c-erbB2 monoclonal antibody was purchased from Neomarkers. All other reagents were of analytical grade. 2-Aminoethoxydiphenyl borate (2-APB) was a gift from Prof. Jianwen Chen (The National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, China).

Cell culture

The MCF-7 human breast cancer cell line was kindly provided by Prof. Jianwen Chen. SK-BR-3 was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (PAA) at 37 °C in a humidified 5% CO_2 incubator.

$[Ca^{2+}]_i$ measurements

The cells (2×10^5 cells) were planted at low density in complete medium for more than 24 h on glass bottom dishes and were starved for 4–6 h in serum-free DMEM before the experiments. $[Ca^{2+}]_i$ was monitored with dual excitation wavelength fluorescence microscopy using Fura-2/AM. In brief, cells were loaded with Fura-2/AM (1 μM) by incubation for 40 min in 5% CO_2 incubator in Ca^{2+} buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glycose, 10 mM Hepes, pH 7.4) and then rinsed with nominally Ca^{2+}-free buffer (145 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 10 mM glycose, 10 mM Hepes, 0.01 mM EGTA, pH 7.4) no less than 20 min to remove extracellular free calcium. Fura-2/AM was excited with light from a mercury lamp alternately filtered to 340 or 380 nm. In a 37 °C environmental chamber, the fluorescence images of the attached cells on the bottom glass (40× objective) were captured every 5 s at emission of 510 nm on an Nikon Diaphot 300 inverted microscope equipped with AquaCosmos Microscopic Image Acquisition. Analysis system was provided by Hamamatsu Photonics K.K. (Japan). The digitized fluorescence ratio (R_{340}/R_{380}) images of the cells and the kinetic change of the ratio in each cell were processed on line on a PC computer. In the imaging system used in this investigation, the excitation light illuminates cells only for very short time (112 ms) during acquisition of each image, but was shut down before next imaging (5 s apart). Therefore, the photobleaching of the Ca^{2+} indicator is negligible.

Mn^{2+} quenching measurements

The rate of Mn^{2+} entry, as measured by the quenching of cellular Fura-2/AM fluorescence after addition of Mn^{2+} (0.2 mM $MnCl_2$), has been used as a measure of store-operated Ca^{2+} influx. The cells were loaded with Fura-2/AM in Ca^{2+} buffer as described above and then rinsed with nominally Ca^{2+}-free buffer no less than 20 min. The fluorescence was excited at 360 nm and measured at 510 nm. The attached cells on the bottom glass were stimulated by agonists before Mn^{2+} addition. The slope of Mn^{2+} entry-induced reduction of Fura-2/AM fluorescence initiated by addition of Tg or HRGβ was estimated as described by Cohen et al. [24]. The initial slopes of the linear decline in Fura-2 fluorescence were during the 30 s immediately after addition of Mn^{2+}.

RNA interference (RNAi)

c-erbB2-siRNA was as follows: sense: 5′-GGAGCUGGCGGCCUUCAUG, antisense: 5′-GCACAAGGCCGCCAGCUCC; negative control siRNA was as follows: sense: 5′-AAUAGUGUAUACGGCAUGC, antisense: 5′-CGAUGCCGUAUACACUAUU. One day before transfection, cells (2×10^5 cells) were plated in 1 ml of growth medium so that they will be 30–50% confluent at the time of transfection. Transfection of 100 nM siRNA duplexes was carried out using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Seventy two hours after transfection, various designated experiments were performed.

Western blotting

Cells were washed twice with cold PBS and then extracted in 30 μl of RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF) at 4 °C for 30 min. The solution was centrifuged at 10,000*g* for 10 min and then the supernatant containing protein was collected.

Protein was separated by 10% SDS–PAGE (20 μg total protein loaded/sample) and electrophoretically transferred, for 1.5 h at 0.8 mA/cm^2, onto polyvinylidene difluoride (PVDF) membrane (Amersham) for subsequent probing. Blots were blocked overnight with 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST), and membranes were incubated for 2 h with primary antibodies. The expression of β-actin was used as loading control. The antibodies used included β-actin (Santa Cruz Biotechnology, sc-47778), c-erbB2 (Neomarkers, MS-1350-P0). The membranes then were incubated for 1 h with HRP-conjugated secondary antibody. Immunocomplexes were visualized with an ECL kit (Pierce). The density of bands was quantitated by using Alphalmager 2200 analysis software.

Statistical analysis

Experimental data were presented as means ± standard deviation of the mean. The data are from at least three different batches of cells. Statistical analysis was made using the Student's *t*-test. $P < 0.05$ was considered to be significant for a difference.

Results

Effect of HRGβ on $[Ca^{2+}]_i$ in human breast cancer with different expression levels of c-erbB2

The pattern of Ca^{2+} mobilization induced by agonist is usually divided into two phases [1,2]. In our experiments, as shown in Fig. 1A, in the absence of extracellular Ca^{2+}, stimulation of MCF-7 cells with 10 nM HRGβ induced a rapid rise in $[Ca^{2+}]_i$ and then gradually decayed toward the baseline. Further replenishment of Ca^{2+} (2 mM) to this solution induced a sustained rise in $[Ca^{2+}]_i$ (Fig. 1A , solid line). HRGβ-induced changes of $[Ca^{2+}]_i$ in MCF-7 involved Ca^{2+} release from the internal Ca^{2+} stores and subsequent Ca^{2+} influx from external source. In the absence of HRGβ, 2 mM Ca^{2+} failed to increase $[Ca^{2+}]_i$ in MCF-7 (Fig. 1A, dotted line). However, in SK-BR-3, 2 mM Ca^{2+} resulted to Ca^{2+} entry even without HRGβ stimulation (Fig. 1B, panel a, dotted line). Interestingly, addition of HRGβ had no effect on Ca^{2+} rapid rise from ER in the cells (Fig. 1B, panel a, solid line). Replacing the bathing solution with 2 mM Ca^{2+} solution, the following peak of external Ca^{2+} entry was enhanced to a similar altitude to the control cells which was in the absence of HRGβ (Fig. 1B, panel a). Our results indicated that HRGβ failed to evoke further increase of $[Ca^{2+}]_i$ in response to addition of Ca^{2+} in SK-BR-3. The above two cell types (MCF-7 and SK-BR-3) showed a significantly different $[Ca^{2+}]_i$ signal in response to addition of HRGβ or Ca^{2+}.

It is known that SK-BR-3 has a high c-erbB2 level in contrast to MCF-7 [23]. To investigate the role of c-erbB2 receptor in the regulation of $[Ca^{2+}]_i$ signal, we employed RNA interference (RNAi) technology to reduce expression level of c-erbB2 in SK-BR-3 (Fig. 1C). The specific siRNA suppressed the synthesis of c-erbB2 protein by 76% (Fig. 1C, panel b). Thus, siRNA treated SK-BR-3 cells had the similar amount of c-erbB2 as MCF-7 cells [23]. In the c-erbB2-siRNA treated SK-BR-3 with HRGβ, an initial rapid rise from ER was observed and Ca^{2+} influx was significantly increased compared to that of SK-BR-3 (Fig. 1B, panel b, solid line and panel a, solid line). Unlike SK-BR-3, the addition of Ca^{2+} to c-erbB2-siRNA treated SK-BR-3 did not exhibit Ca^{2+} entry in the absence of HRGβ (Fig. 1B, panel b, dotted lines). The c-erbB2-siRNA treated SK-BR-3 with low c-erbB2 level showed similar $[Ca^{2+}]_i$ signal pattern to MCF-7 (Fig. 1B, panel b and A).These results suggested that c-erbB2 expression level might be responsible for pattern of Ca^{2+} mobilization induced by HRGβ.

SOC was responsible for Ca^{2+} entry in the presence or in the absence of HRGβ

Our observation was based on two inhibitors: lanthanide (La^{3+}) and 2-aminoethoxydiphenyl borate (2-APB), which are widely used as blockers for SOC [25–28]. After the initial rise stimulated by HRGβ, Ca^{2+} entry triggered by addition of 2 mM Ca^{2+} was abolished by 2 μM La^{3+} or 20 μM 2-APB, which made $[Ca^{2+}]_i$ signal return to resting levels in MCF-7 (Figs. 2A and B) and c-erbB2-siRNA treated SK-BR-3 (Figs. 2C, panel b and D, panel b). In both HRGβ-treated (solid lines) and untreated (dotted lines) SK-BR-3 cells, La^{3+} or 2-APB was also effective, completely reducing peak HRGβ-induced Ca^{2+} entry. (Figs. 2C, panel a and D, panel a). Taken together, the results obtained from La^{3+} or 2-APB experiments indicated that Ca^{2+} entry in the presence or in the absence of HRGβ was via SOC.

To further demonstrated that HRGβ-evoked Ca^{2+} entry attributed to store-operated Ca^{2+} channel (SOC), the rate of Mn^{2+} entry, as measured by the quenching of cellular Fura-2/AM fluorescence, has been used to estimate store-operated Ca^{2+} entry [29]. Thapsigargin (Tg), a specific inhibitor of the Ca^{2+} ER pump, is used most often to activate Ca^{2+} influx via SOC in various cells [1,2]. Compared with the rate of Mn^{2+} quenching without any agonists (Fig. 3A, panel a), stimulation of the cells with Tg or HRGβ before Mn^{2+} addition dramatically increased the rate of Mn^{2+} entry in MCF-7 (Fig. 3A, panel b and panel c). It was clearly shown that both of Tg and HRGβ activated store-operated Ca^{2+} entry in MCF-7. In SK-BR-3, compared with the basal Mn^{2+} quenching signal recorded in the absence of any agonists (Fig. 3B, panel a), Tg dramatically increased the rate of Mn^{2+} entry (Fig. 3B, panel b). However, the rate of Mn^{2+} entry in the presence of HRGβ (Fig. 3B, panel c) was similar to that of the basal Mn^{2+} quenching signal (Fig. 3B, panel a), indicating that addition of HRGβ did not lead to further activation of SOC in SK-BR-3. Interestingly, in c-erbB2-siRNA treated SK-BR-3, HRGβ addition reactivated the dramatic Mn^{2+} entry compared to the resting state (Fig. 3C, panel a and panel c). In fact, RNAi to decrease c-erbB2 level in SK-BR-3 resulted in the similar Mn^{2+} quenching signals to that of MCF-7 either in the presence or in the absence of agonists (Figs. 3A and C). In Fig. 3D, the basal Mn^{2+} quenching signal of SK-BR-3 was more dramatic than that of MCF-7 and c-erbB2-siRNA treated SK-BR-3. The results suggested that

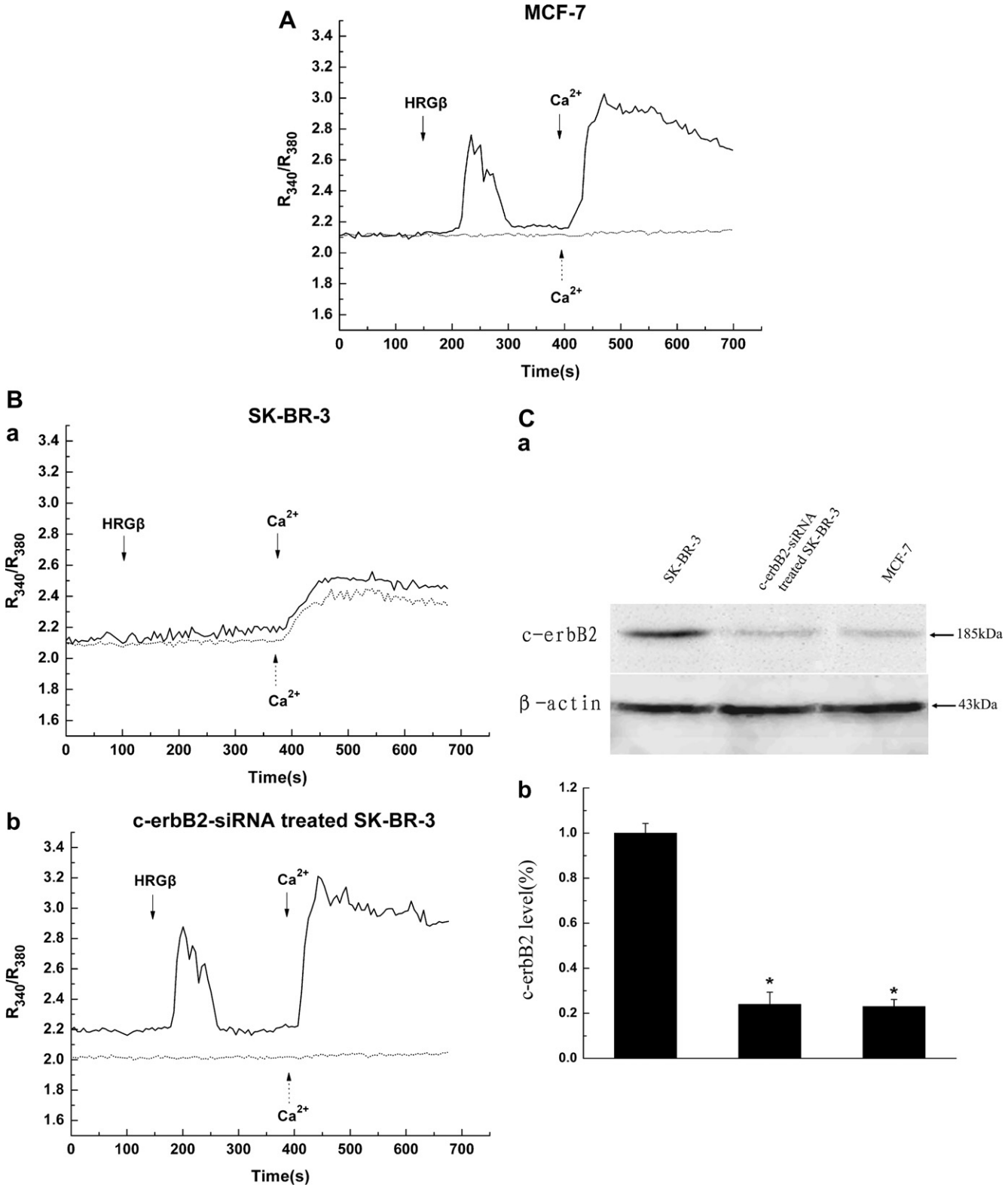

Fig. 1. Effect of HRGβ on the $[Ca^{2+}]_i$. MCF-7 (A), SK-BR-3 (B, panel a) or c-erbB2-siRNA treated SK-BR-3 (B, panel b) were treated (solid lines) and untreated (dotted lines) with HRGβ (10 nM) as indicated. Extracellular Ca^{2+} (2 mM) was replenished as indicated in both HRGβ-treated and untreated cells. All kinetic curves were the average of those observed in 30 cells from 3 experiments. (C) Western blotting of cell extracts (SK-BR-3, c-erbB2-siRNA treated SK-BR-3 and MCF-7) were subjected to 10% SDS–PAGE, then probed using polyclonal antibodies specific for c-erbB2 and β-actin was a control for protein loading (C, panel a) as described in Materials and methods. (C, panel b) is summary data of percent change in c-erbB2 expression level of three cell types. The SK-BR-3 was normalized to 100%. The bar graphs shown in this figure were representative of 4 independent experiments. The asterisks indicate that results are statistically significant when compared to the c-erbB2 expression level in SK-BR-3 ($^*P < 0.05$). Values represent averages of four determinations ±SD.

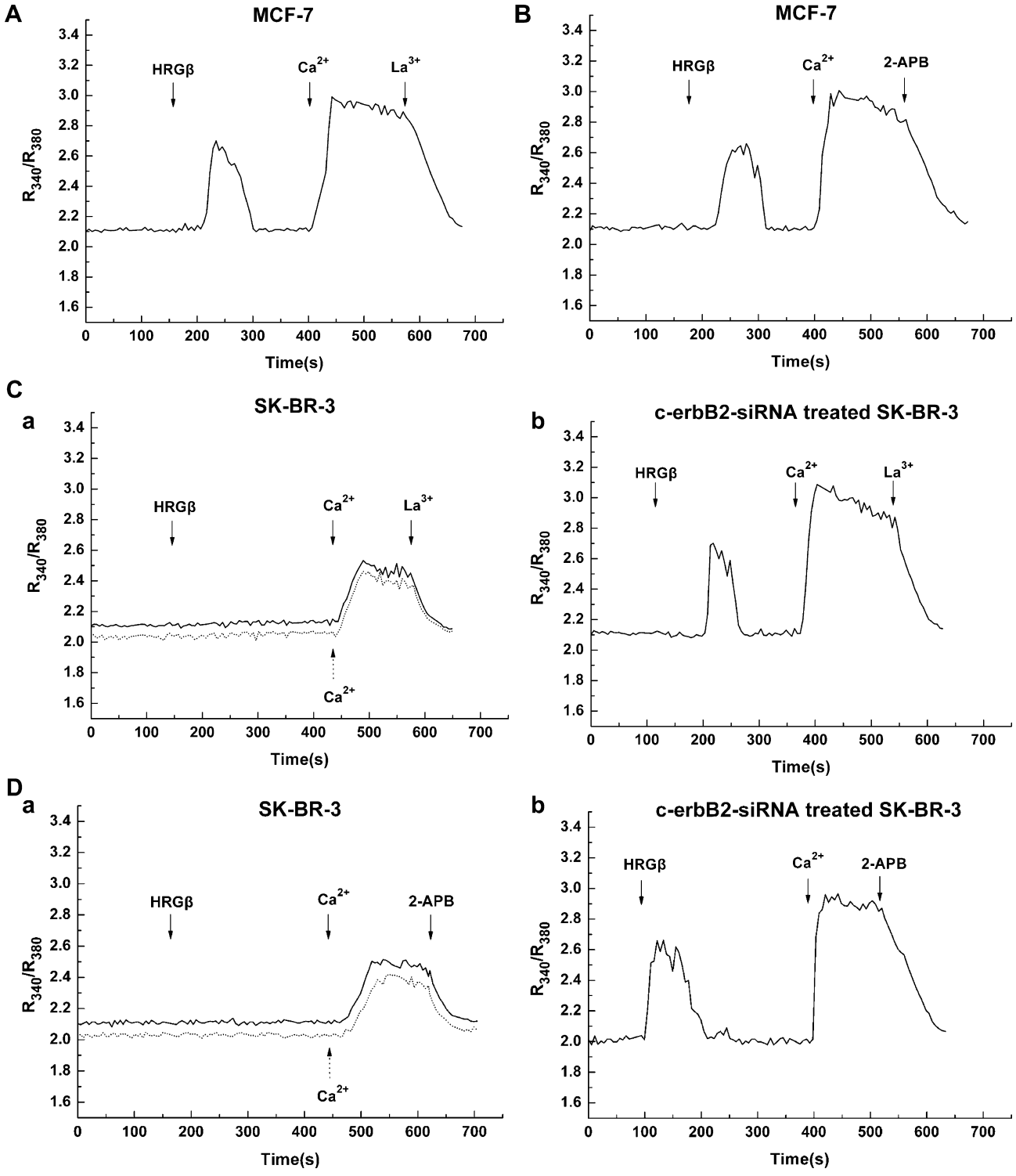

Fig. 2. Effect of La^{3+} (A and C) or 2-APB (B and D) on HRGβ-induced Ca^{2+} influx. MCF-7 (A), SK-BR-3 (C, panel a) and c-erbB2-siRNA treated SK-BR-3 (C, panel b) were stimulated with HRGβ (10 nM), followed by subsequent addition of 2 mM Ca^{2+} and 2 μM La^{3+}, respectively, which were indicated as arrows. MCF-7 (B), SK-BR-3 (D, panel a) and c-erbB2-siRNA treated SK-BR-3 (D, panel b) were stimulated with HRGβ (10 nM), followed by subsequent addition of 2 mM Ca^{2+} and 20 μM 2-APB, respectively, which were indicated as arrows. SK-BR-3 (C, panel a and D, panel a) were treated with Ca^{2+} (2 mM) in the absence of HRGβ (dotted lines), which were indicated as arrows. All kinetic curves were the average of those observed in 41 cells from 5 experiments.

although MCF-7, SK-BR-3 and c-erbB2-siRNA treated SK-BR-3 had SOC components, the three cell types exhibited significantly different Mn^{2+} entry responses to HRGβ.

HRGβ-induced Ca^{2+} mobilization was dependent on PLC

To assess whether Ca^{2+} mobilization in the presence of HRGβ were mediated by PLC in MCF-7, SK-BR-3 and

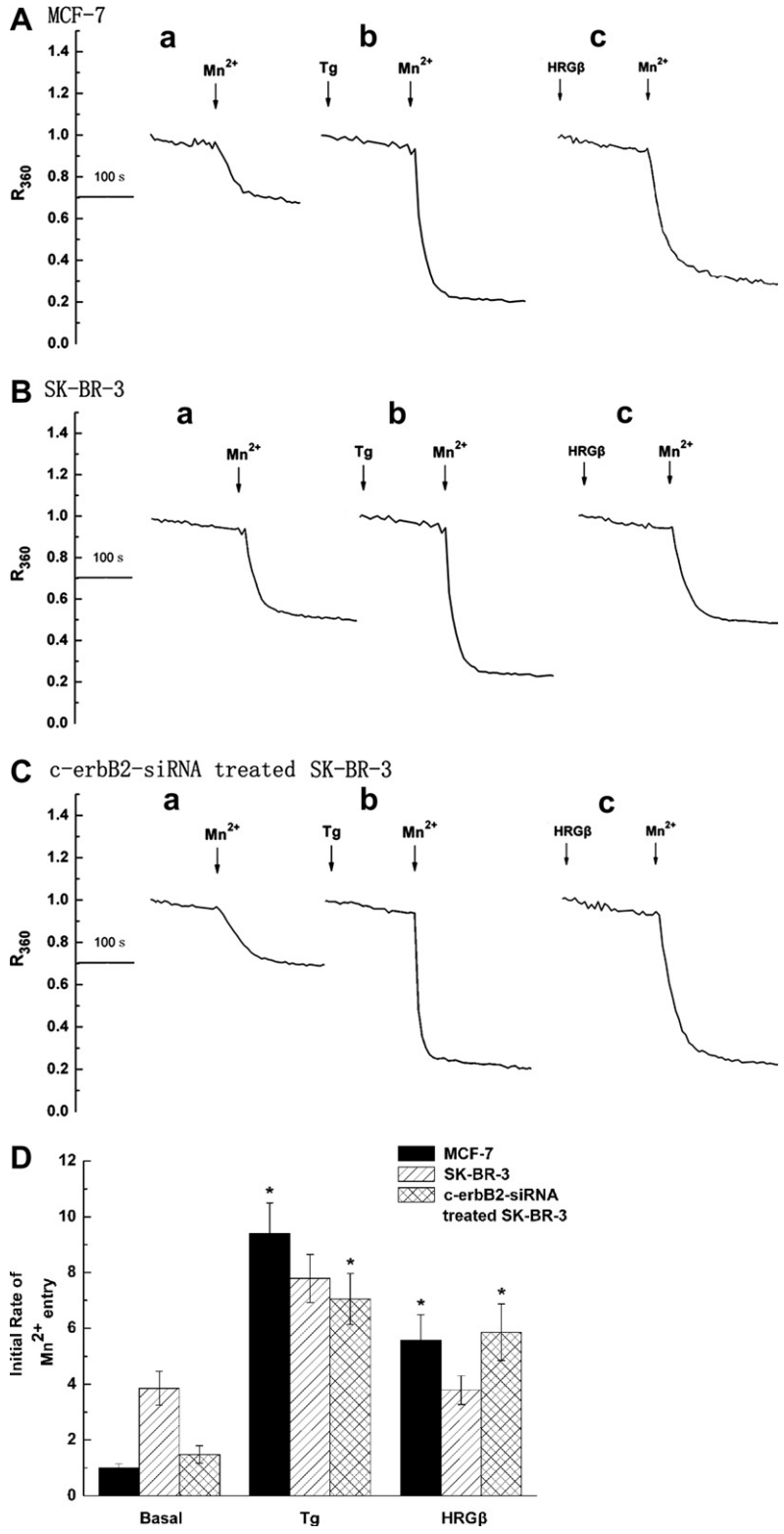

Fig. 3. Effect of Tg or HRGβ on Mn^{2+} entry. MCF-7 (A), SK-BR-3 (B) and c-erbB2-siRNA treated SK-BR-3 (C) were either unstimulated or stimulated in nominally Ca^{2+}-free buffer with 10 nM HRGβ (c) or 10 nM Tg (b) as indicated by the first vertical arrow in each trace. Quench of fluorescence recorded as Mn^{2+} (0.2 mM) was added (indicated by the single vertical arrow in a and the second vertical arrow in (b) and (c)). All kinetic curves were the average of those observed in 35 cells from 4 experiments. (D) Summary of rates of Mn^{2+} entry, normalized to the rate under basal conditions of MCF-7. The bar graphs represent the basal rate without any agonists (a) and the rate with Tg (b) or HRGβ (c) in MCF-7, SK-BR-3 and c-erbB2-siRNA treated SK-BR-3 in nominally Ca^{2+}-free buffer. The asterisks indicate that results are statistically significant when compared to the basal Mn^{2+} quenching signal (a) ($^{*}P < 0.05$). Values represent averages of four determinations ±SD.

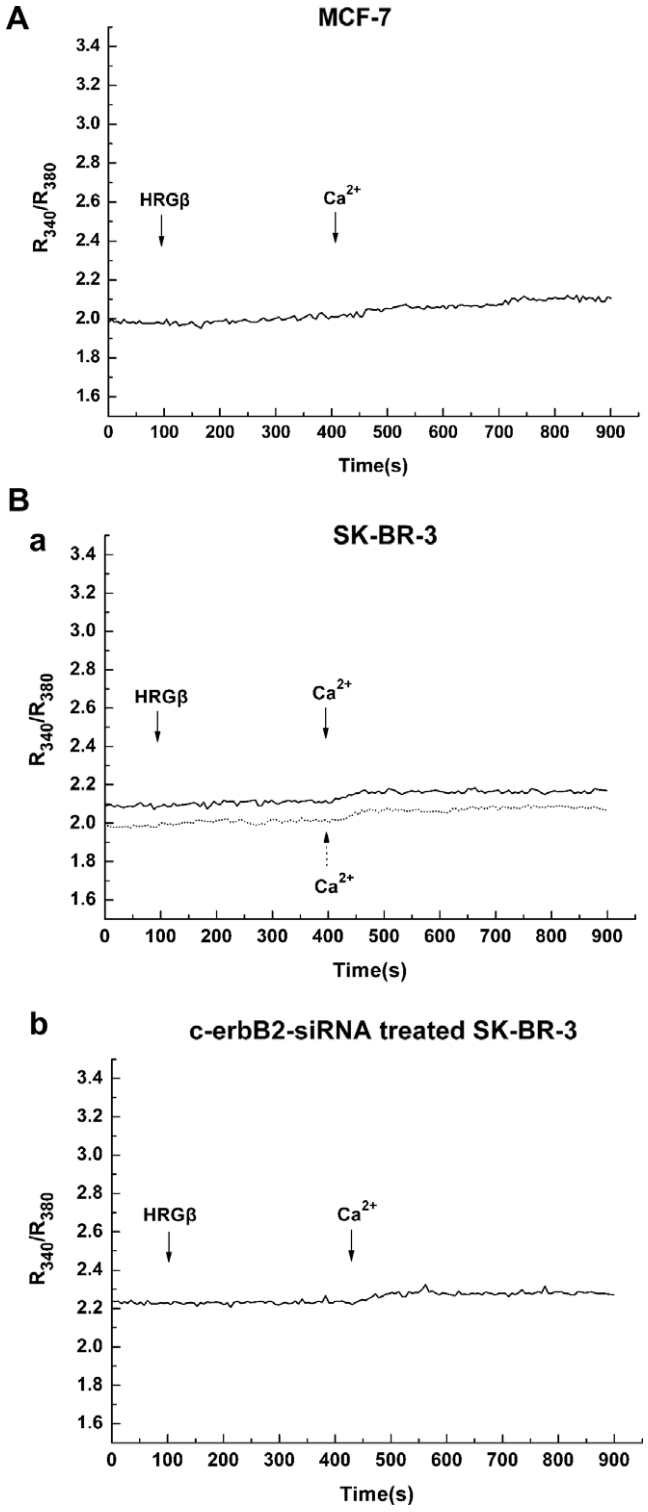

Fig. 4. Effect of the PLC inhibitor U73122 on HRGβ-induced Ca^{2+} release and Ca^{2+} entry. MCF-7 (A), SK-BR-3 (B, panel a) or c-erbB2-siRNA treated SK-BR-3 (B, panel b) were treated (solid lines) with HRGβ (10 nM) as indicated. SK-BR-3 cells (B, panel a) were treated with Ca^{2+} (2 mM) in the absence of HRGβ as control (dotted line). Extracellular Ca^{2+} (2 mM) was replenished as indicated in both HRGβ-treated and untreated cells. Before stimulation of HRGβ, cells were preincubated with U73122 (10 μM) at room temperature for 10 min to block subsequent Ca^{2+} release and Ca^{2+} entry. All kinetic curves were the average of those observed in 30 cells from 3 experiments.

c-erbB2-siRNA treated SK-BR-3, a membrane-permeable PLC inhibitor U73122 was used [30]. A 10 min preincubation of cells with 10 μM U73122 has been demonstrated to fully and irreversibly prevent PLC activation upon agonist stimulation in a variety of cell types. In our experiments, applications of U73122 to MCF-7 (Fig. 4A) and c-erbB2-siRNA treated SK-BR-3 (Fig. 4B, panel b) were sufficient to depress the HRGβ-induced Ca^{2+} release from ER in the absence of extracellular Ca^{2+}, and subsequently reduced Ca^{2+} influx to the baseline after external Ca^{2+} repletion. In SK-BR-3, in the presence or in the absence of HRGβ, Ca^{2+} entry via SOC was almost completely abolished by U73122 (Fig. 4B, panel a). These data suggested that Ca^{2+} release induced by HRGβ and Ca^{2+} influx via SOC in the presence or in the absence of HRGβ were dependent on the activation of PLC.

Discussion

In the present study, we provide evidence that addition of HRGβ was able to activate intracellular Ca^{2+} increase via SOC in the cells with low c-erbB2 level (MCF-7 and c-erbB2-siRNA treated SK-BR-3), whereas the cells with high c-erbB2 level (SK-BR-3) was not sensitive to HRGβ. In the absence of HRGβ, a constitutive activation of SOC was observed in the cells with high c-erbB2 level rather than in the cells with low c-erbB2 level. The implication of our study was that the different $[Ca^{2+}]_i$ signal with or without HRGβ correlates the c-erbB2 expression level in human breast cancer.

In cells with low expression level of c-erbB2 (MCF-7 and c-erbB2-siRNA SK-BR-3), HRGβ activated Ca^{2+} release from internal stores and Ca^{2+} influx across the plasma membrane. HRGβ-activated Ca^{2+} entry was most likely mediated by SOC in the two cell types. Our observation was based on three lines of evidence. First, the HRGβ-activated Ca^{2+} mobilization was dependent on activation of PLC, because PLC inhibitor U73122 completely inhibited an increase of $[Ca^{2+}]_i$ in MCF-7 (Fig. 4A) and c-erbB2-siRNA SK-BR-3 (Fig. 4B, panel b). Ca^{2+} release and Ca^{2+} entry via a PLC-dependent pathway are consistent with the characteristic of SOC [3–5]. Second, to further determine whether HRGβ was capable of activating SOC, we demonstrated that HRGβ-induced Ca^{2+} entry were abolished by La^{3+} or 2-APB, reliable SOC blockers (Figs. 2A, B, C, panel b and D, panel b). Third, HRGβ dramatically enhanced the rate of Mn^{2+} entry, which was similar to the action of Tg in MCF-7 (Fig. 3A) and c-erbB2-siRNA treated SK-BR-3 (Fig. 3C). It is not strange that the c-erbB2 downregulation conferred SK-BR-3 a phenotype that was similar to that of MCF-7. Previous study showed that, using RNA interference technology to downregulate the amount of EGFR in SK-BR-3 to similar level in BT474, SK-BR-3 exhibited similar erbB-receptor phenotype and responsiveness to growth factors to that of BT474 [31].

In SK-BR-3 cells with high expression level of c-erbB2, HRGβ could not trigger Ca^{2+} release and Ca^{2+} entry via SOC. In our experiments, SK-BR-3 cells with HRGβ

showed a similar altitude of Ca^{2+} entry to the cells without HRGβ when 2 mM Ca^{2+} solution was added (Fig. 1B, panel a), which was significantly lower than that of c-erbB2-siRNA treated SK-BR-3 (Fig. 1B, panel b). The above results are consistent with the results obtained from the rate of Mn^{2+} quenching experiments showing that the rate in the presence of HRGβ (Fig. 3B, panel c) was similar to that of the basal quenching signal (Fig. 3B, panel a) in SK-BR-3. In addition, in c-erbB2-siRNA treated SK-BR-3, the rate of Mn^{2+} quenching with HRGβ was more dramatic than that of SK-BR-3 (Fig. 3D). Brockhoff et al. previously demonstrated that, in SK-BR-3, HRGβ incubation for 0.5 h failed to induce further interaction between c-erbB2 and c-erbB3 [22]. In our experiments, it is not difficult to conclude that addition of HRGβ do not result in more c-erbB2/c-erbB3 interaction and subsequently fail to activate further $[Ca^{2+}]_i$ signal. Thus, the constitutive activation of erbB-receptor interaction was considered to lead to resistance to HRGβ in a certain extent.

The cells with various expression levels of c-erbB2 exhibited the very different $[Ca^{2+}]_i$ signals not only in the presence of HRGβ but also in the absence of HRGβ. MCF-7 cells and c-erbB2-siRNA treated SK-BR-3 cells have low c-erbB2 receptor level, thus the two cell types might lack a constitutive c-erbB2/c-erbB3 interaction in the absence of HRGβ. In support, the constitutive activation of SOC in the absence of HRGβ was not observed in MCF-7 (Fig. 1A, dotted line) and c-erbB2-siRNA treated SK-BR-3 (Fig. 1B, panel b, dotted line). However, in SK-BR-3, which overexpress c-erbB2, the likelihood of heterodimerization for the c-erbB2 receptor was also high in the resting state. Consequently, high c-erbB2 receptor level in SK-BR-3 resulted in interaction with c-erbB3 even without ligand addition. In the cells, constitutive c-erbB2/c-erbB3 interaction has been found to constitutively activate PKB/Akt and MAPK signaling pathway [22]. In the present study, PLC signaling pathway was activated without ligand addition and subsequently mediated activation of SOC. In our experiments, blocking effect of La^{3+} or 2-APB on SK-BR-3 in the absence of HRGβ showed that the cells constitutively activated Ca^{2+} entry via SOC (Figs. 2C, panel a, dotted line and D, panel a, dotted line). In addition, the data from Mn^{2+} quenching experiments showed that the basal Mn^{2+} quenching signal of SK-BR-3 was more dramatic than that of MCF-7 and c-erbB2-siRNA treated SK-BR-3 (Fig. 3D). The present study provides evidence that the constitutive activation is due to the difference in c-erbB2/c-erbB3 interaction along with c-erbB2 receptor level. Taken together, these results lead us to propose that the HRGβ-induced different $[Ca^{2+}]_i$ signals in the cells with various expression level of c-erbB2 are most likely attributed to c-erbB2/c-erbB3 interaction in the resting state.

In SK-BR-3 cells, HRGβ failed to trigger measurable Ca^{2+} release. How should we interpret the paradoxical results that in cells with high expression level of c-erbB2 (SK-BR-3), Ca^{2+} entry without HRGβ addition was via SOC but store depletion was not observed? It is noteworthy to mention that PLC has been proposed to play a critical role in SOC activation. In the previous study, PLC-γ was considered as a molecular to associate with Ca^{2+} entry channel or the related components. Through its SH3 domain, PLC-γ binds directly to the transient receptor potential (TRP) channels, which are defined as components of SOC [26]. Patterson et al. proposed that the role of PLC-γ is independent of production of IP_3, because its catalytic function was not required. Thus, SOC was activated not only by lowering of Ca^{2+} content in ER, but by a receptor-mediated activation of PLC-γ. In accordance, Li et al. found that in human glomerular mesangial cells, Ca^{2+} release from ER was not required in the process of EGF-activated SOC [7]. Our experiments on SK-BR-3 exhibited that constitutive c-erbB2/c-erbB3 interaction might result in interaction between PLC-γ and Ca^{2+} entry channel and trigger mild Ca^{2+} entry. In support, we found that Ca^{2+} entry via SOC was almost completely abolished by specific PLC inhibitor (U73122) in the absence of HRGβ in SK-BR-3 (Fig. 4B, panel a, dotted line). Compared to the results that HRGβ-activated Ca^{2+} release and Ca^{2+} entry in the low c-erbB2 cells (MCF-7 and c-erbB2-siRNA treated SK-BR-3), Ca^{2+} entry through SOC might via distinct mechanism in the cells high c-erbB2 level (SK-BR-3).

Recently, Diermeier et al. demonstrated that in SK-BR-3 cells, signal transmission over the cell membrane is defined by erbB-receptor coexpression rather than by c-erbB2 overexpression alone [31]. In their study, the different efficiency of a therapeutic antibody is determined by a different and cell specific EGFR/c-erbB2 profile which is activated by EGF, because the antibodies have different capacity to specifically disrupt erbB-receptor homo- and heteroassociation and thereby convey distinct therapeutical effectiveness. HRGβ-activated c-erbB2/c-erbB3 heterodimerization appears to be an important target for therapy. Our experiments focus on initiating signals of downstream signal transduction mediated by c-erbB2/c-erbB3 interaction, that is cytosolic Ca^{2+} mobilization by PLC-dependent signaling pathway. The study addressing the relationship of Ca^{2+} mobilization mechanism triggered by HRGβ and c-erbB2/c-erbB3 coexpression profile provides an essential basis for understanding in diversified responses to erbB-receptor-based therapies in the cells with different c-erbB2 expression level in human breast cancer cells.

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