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EGF receptor-mediated intracellular calcium increase in human hepatoma BEL-7404 cells[1]

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ABSTRACT

Epidermal growth factor (EGF) induced intracellular free calcium ($[Ca^{2+}]_i$) response was studied in fura-2- or fluo-3-loaded human hepatoma cells of BEL-7404 cell line. Single cell $[Ca^{2+}]_i$ analysis and $[Ca^{2+}]_i$ measurement in cell populations revealed that EGF triggered a rapid $[Ca^{2+}]_i$ increase in the dose-dependent and time- dependent manner. Pretreatment of cells with an endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, thapsigargin (TG) at 100 nM concentration for 20 min, completely abolished EGF-induced $[Ca^{2+}]_i$ increase, and chelating extracellular calcium by excess EGTA partially inhibited the increase. Furthermore, the expression of antisense EGF receptor sequence in BEL-7404 cells suppressed the $[Ca^{2+}]_i$ response to EGF. The results suggest that EGF receptor-mediated $[Ca^{2+}]_i$ increase in the human hepatoma ceils is essentially dependent on the Ca^{2+} storage in ER.

Key words: *EGF receptor, calcium, thapsigargin, human hepatoma cells.*

INTRODUCTION

Epidermal growth factor (EGF) and its receptor are key regulatory components

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of cell growth and differentiation in a variety of cell types[1]. The EGF receptor tyrosine kinase- mediated signal transduction pathways have been suggested to play the pivotal role under either physiological condition or pathological condition, for example, cancer. Among the multiple signal transduction pathways, EGF induces the intracellular free calcium ($[Ca^{2+}]_i$) increase in several cell types, but the underlying mechanism is so far unresolved. In some situation, the $[Ca^{2+}]_i$ response has been considered to be linked to the hydrolysis of polyphosphoinositides by phospholipase C_γ, which is activated as a result of phosphorylation on tyrosine residues by the EGF receptor tyrosine kinase[2]. As a consequence, the second messengers 1,2-diacylglycerol and inositol-1, 4, 5-trisphosphate are produced. The former activates protein kinase C and the latter releases Ca^{2+} from internal stores in endoplasmic reticulum[3, 4]. Besides the induction of internal Ca^{2+} release, EGF induces the Ca^{2+} influx through the plasma membrane in some cell types. In these cells, the activation of the Ca^{2+} channels by EGF stimulation is independent of phospholipase C, but mediated by the activation of phospholipase A_2[5]. In the previous studies, we have demonstrated the EGF receptor gene expression and mitogenic effects of EGF in cells of a human hepatoma cell line BEL-7404[6]. We have recently obtained a cell clone, which constitutively expresses the antisense EGF receptor sequence, from BEL-7404 cells[7]. These cell lines might be useful for studying the signalling of EGF.

In the present study, the effects of EGF on $[Ca^{2+}]_i$ were examined in BEL-7404 cells and its derivatives by using the $[Ca^{2+}]_i$ measurement in cell populations combined with single-cell $[Ca^{2+}]_i$ analysis. The dependency of EGF-induced $[Ca^{2+}]_i$ responses on extracellular Ca^{2+} concentration and intracellular Ca^{2+} storages was also studied.

MATERIALS AND METHODS

Materials

Fluo-3/AM, fura-2/AM, thapsigargin were perchased from Sigma (St. Louis, MO, USA). EGF was from Gibco BRL (Gaithersburg, MD, USA). All other chemicals were from commercial sources.

Cell Culture

Human hepatoma cell line BEL-7404 cells and an antisense EGF receptor-expressed cell clone derived from BEL-7404 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 13 % fetal calf serum, 100 μ/ml of penicillin and 100 μg/ml of streptomycin in 5 % CO_2 incubator. After being cultured at 37 °C for 48 h, the cells were kept in the serum-free medium for 24 h before the determinations unless otherwise indicated.

Measurement of intracellular free Ca^{2+} concentration

For single-cell $[Ca^{2+}]_i$ microspectrofluorimetry, cells were plated at a density of 1×10^4 cells/well on the 6-well Nunclon plate inserted with sterile glass coverslips. After the cells were cultured on the condition indicated above, the coverslips were incubated with assay buffer (NaCl 125 m*M*, KCl 5 m*M*, $CaCl_2$ 1 m*M*, $MgCl_2$ 1 m*M*, Glucose 10 m*M*, HEPES 25 m*M*, pH 7.05 and 0.1% bovine serum albumin) containing fura-2/AM (5 μM) at 37 °C for 45 min. At the end of the incubation, the

coverslips were mounted on the recording chamber of a microscope equipped for microfluorimetry as described previovusly[8].Fluorescence intensity was obtained with dual excitation wavelengths set at 350 and 380 nm and emission wavelength at 510 nm,respectively $[Ca^{2+}]_i$ was valued from the 350 nm/380 nm fluorescence ratios.

For measurement of $[Ca^{2+}]_i$ in cell populations, the cells were incubated with the assay buffer containing fluo-3/AM (5 μM) at 37°C for 30 min. At the end of the loading, the cells were detached by the treatment with 0.125 % trypsin in Ca^{2+}-free and Mg^{2+}-free Hank's solution for 3 min. After centrifugation at 250 × g for 3 min, the cell pellet was resuspended in 1 ml of the assay buffer. The fluorescence of individual cells was measured using a FACStar Plus flow cytometer (Becton and Dickinson, CA, USA). For each assay, 2,500 cells were examined and the mean value was taken. All the data were recorded and analyzed using specific FACStar research software. The change of $[Ca^{2+}]_i$ was expressed as △F/F (%), the percentage of actual fluorescence to the control fluorescence level in unstimulated state.

RESULTS

1. EGF induced $[Ca^{2+}]_i$ increase in human hepatoma BEL-7404 cells

Measurement of $[Ca^{2+}]_i$ in cell populations loaded with fluorescent indicator fluo-3 demonstrated that EGF elicited the increase of fluorescence intensity, which reflected the increase of $[Ca^{2+}]_i$, in a dose-dependent manner, with the maximal dose around 300 ng/ml (Fig 1). Different doses (from 3 ng/ml to 300 ng/ml) of EGF produced different magnitudes but similar kinetics of Ca^{2+} signals (see Fig 1, inset). Therefore, in the following experiments, the maximal dose (300 ng/ml) of EGF was used unless otherwise indicated. Single-cell $[Ca^{2+}]_i$ measurement in fura-2-1oaded cells revealed that EGF (300 ng/ml) induced the transient $[Ca^{2+}]_i$ increase in BEL-7404 cells (Fig 2). The peak of $[Ca^{2+}]_i$ response was observed around 1 min after EGF application, and the ratio (350 nm/380 nm) increased about 3-fold. Then, the $[Ca^{2+}]_i$ level rapidly decreased within 2 min of the stimulation. As shown in Fig 3 a, EGF (300 ng /ml) induced the rapid increase of $[Ca^{2+}]_i$ in cell populations, which was initially identical to that obtained in single-cell $[Ca^{2+}]_i$ measurement except a slow falling phase following the $[Ca^{2+}]_i$ peak.

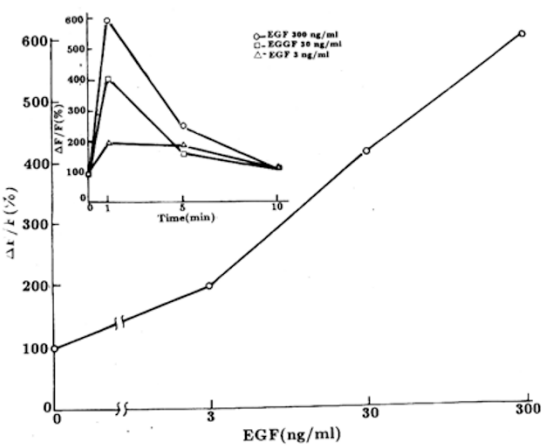


Fig 1. Dose-dependence increase of EGF-induced $[Ca^{2+}]_i$ in fluo-3-loaded BEL7404 cells with flow cytometry. $[Ca^{2+}]_i$ levels were measured in cell populations after the application of indicated doses of EGF for 1 min. Inset, $[Ca^{2+}]_i$ levels were recorded in cell populations after application of different doses of EGF (3, 30 and 300 ng/ml, respectively) for indicated periods.

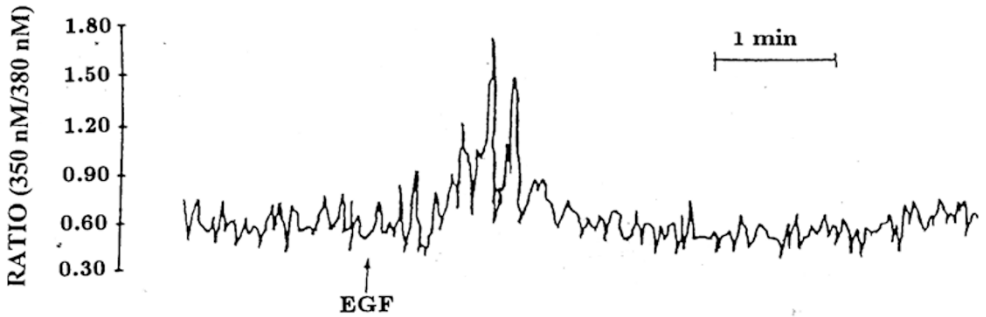
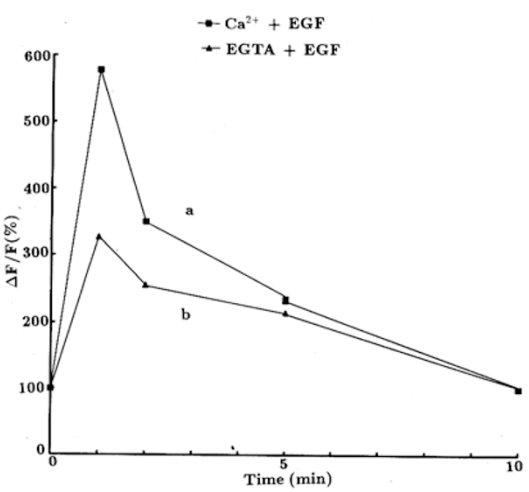


Fig 2. EGF-induced $[Ca^{2+}]_i$ increase in fura-2-1oaded BEL-7404 cells measured by microspectrofluorimetry with single-cell $[Ca^{2+}]_i$ measurement. EGF (300 ng/ml) was added at the point indicated by arrow.

2. Dependency of EGF-induced $[Ca^{2+}]_i$ increase on extracellular Ca^{2+} concentration

As shown in Fig 3 b, in Ca^{2+}-free assay buffer, pretreatment of cells with 1 m*M* EGTA for 3 min partially inhibited the EGF-induced $[Ca^{2+}]_i$ increase, especially in the early stage, for example 1 min after the stimulation, the inhibition was about 50% as compared with the $[Ca^{2+}]_i$ response in 1 mM Ca^{2+}-containing assay buffer. The results suggested the contribution of Ca^{2+} influx to the total $[Ca^{2+}]_i$ increase was triggered b y EGF.

Fig 3. Effect of extracellular Ca^{2+} on EGF-induced $[Ca^{2+}]_i$ increase in fluo-3-1oaded BEL-7404 cells. (a), $[Ca^{2+}]_i$ levels were measured in cell populations after the application of EGF (300 ng/ml) for 1, 2, 5, 10 min respectively, in the presence of 1 m*M* Ca^{2+}. (b), the cells were preincubated in Ca^{2+}-free assay buffer containing 1 m*M* EGTA for 3 min, and then $[Ca^{2+}]_i$ levels were measured in cell populations after the addition of EGF (300 ng/ml) to the above buffer for indicated periods.



3. The effects of thapsigargin on $[Ca^{2+}]_i$ with or without the EGF stimulation

As expected, thapsigargin (TG) at 100 n*M* concentration did produce the pronounced $[Ca^{2+}]_i$ increase in human hepatoma BEL-7404 cells (Fig 4A). In the Ca^{2+}-free assay buffer , TG also induced some $[Ca^{2+}]_i$ increase but to a much less extent (Fig 4B). Interestingly, when 1 m*M* Ca^{2+} was re-added to the above sample, the $[Ca^{2+}]_i$ was rapidly elevated again to the level comparable to the peak of early $[Ca^{2+}]_i$ response in the cells incubated with 100 n*M* TG in 1 m*M* Ca^{2+}-containing assay buffer, which suggested that Ca^{2+} influx could occur as a consequence of the TG- induced Ca^{2+} store depletion.

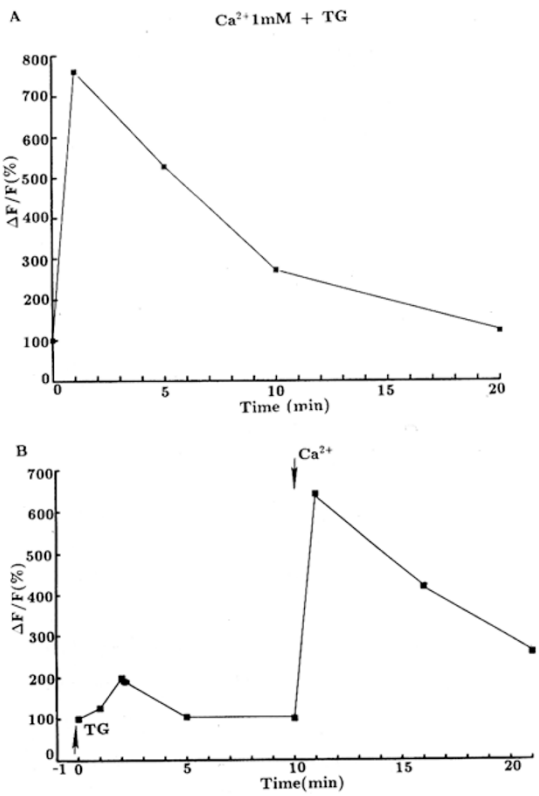


Fig 4. Effects of TG (100 n*M*) on $[Ca^{2+}]_i$ in fluo-3-loaded BEL-7404 cells. $[Ca^{2+}]_i$ levels were measured in cell populations. A, TG was added into the assay buffer containing 1 m*M* Ca^{2+}, and $[Ca^{2+}]_i$ levels were recorded after the application of TG for 1, 5, 10, 20 min, respectively. B, Before stimulation, the cells were washed twice with 1 m*M* EGTA-containing Ca^{2+}-free assay buffer. TG was added into Ca^{2+}-free assay buffer for 10 min, then 1 m*M* Ca^{2+} was re-added into the sample at the point indicated by arrows. Measurements of $[Ca^{2+}]_i$ were performed at the points as shown in the figure.

EGF receptor-mediated calcium increase in hepatoma cells

In order to investigate the contribution of intracellular Ca^{2+} storage to the EGF-induced $[Ca^{2+}]_i$ increase, we did some $[Ca^{2+}]_i$ measurements either through the addition of EGF (300 ng/ml) to suspended human hepatoma BEL-7404 cells pretreated with TG (100 n*M*) for 20 min or through the addition of TG (100 n*M*) to the cells pretreated with EGF (300 ng/ml) for 10 min. The data showed that the pretreatment with maximal dose of EGF (300 ng/ml) for 10 min exhibited no inhibitory effect on TG-induced $[Ca^{2+}]_i$ increase, whereas preincubation with TG (100 n*M* for 20 min) completely inhibited the EGF-induced $[Ca^{2+}]_i$ increase in these cells (Tab 1).

Tab 1. Effects of TG and EGF on $[Ca^{2+}]_i$ in human hepatoma BEL-7404 cells*

Stimulations	△F/F(%,M±S.D.)
1. EGF 300 ng/ml, for1 min	579 ±26
2. TG 100 n*M* for 1 min	761 ± 80
3. EGF 300 ng/ml, for 10 min, then	
a. addition of the buffer for 1 min	101 ± 8
b. addition of TG 100 n*M* for 1 min	706 ± 32
4. TG 100 n*M* for 20 min, then	
a. addition of the buffer for 1 min	120 ± 8
b. addition of EGF 300 ng/ml for 1 min	114 ± 5

* $[Ca^{2+}]_i$ levels were measured in fluo-3-1oaded cell populations as described in "Materials and Methods", and all $[Ca^{2+}]_i$ measurements were performed in the presence of 1 m*M* Ca^{2+}. (1) and (2), $[Ca^{2+}]_i$ levels were recorded after the application of either EGF (300 ng/ml) or TG (100 n*M*) for 1 min. (3), After the preincubation with EGF (300 ng/ml) for 10 min, without washing, the assay buffer (100 μl) was added in (a) and TG (100 n*M*) was added in (b) 1 min before $[Ca^{2+}]_i$ measurements. (4), After the preincubation with TG (100 n*M*) for 20 min, without washing, the assay buffer (100 μl) was added in (a) and EGF (300 ng/ml) was added in (b) 1 min before $[Ca^{2+}]_i$ measurement.

4. Expression of antisense EGF receptor suppressed the EGF-induced $[Ca^{2+}]_i$ increase in human hepatoma BEL-7404 cells

Recently, we have obtained a cell clone, JX-1, constitutively expressing the antisense EGF receptor sequence, and a control cell clone, JX-0, transferred with the vector plasmid in BEL-7404 cells[7]. We found that EGF-induced $[Ca^{2+}]_i$ increase in JX-1 cells were obviously inhibited in comparison with those in control JX-0 cells, although JX-0 cells showed a lower amplitude of $[Ca^{2+}]_i$ increase than that in BEL-7404 cells for an unknown reason. When JX-1 cells were serum-starved for 24 h, the $[Ca^{2+}]_i$ response to EGF stimulation were nearly abolished (Fig 5). Without serum-starvation, the EGF-induced $[Ca^{2+}]_i$ increase in JX-1 cells were inhibited by 29.5% to 50.3% in the presence of different doses of EGF (data not shown).

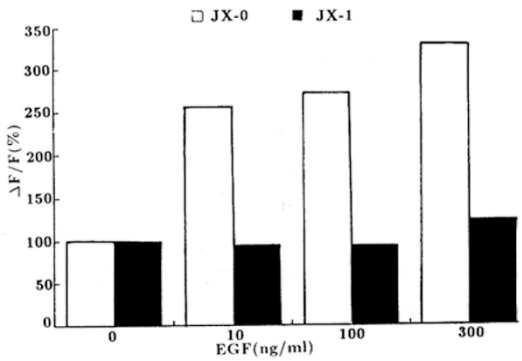


Fig 5. Increase of $[Ca^{2+}]_i$ induced by EGF in fluo-3-1oaded JX-0 and JX-1 cells. The cells were serum-starved for 24 h before the determinations and $[Ca^{2+}]_i$ was recorded after the application of indicated doses of EGF for 1 min.

DISCUSSION

The use of fluorescent indicators sensitive to Ca^{2+} and readily introduced into cells represented a major advance in the measurement of $[Ca^{2+}]_i$. The first of these, quin-2, has been largely superseded by fura-2[9]. Fluo-3, a more recent derivative, has the major advantage of having an excitation spectrum that is visible and thus does not require expensive quartz optics[10]. In the present study, a combination of single-cell $[Ca^{2+}]_i$ analysis with the $[Ca^{2+}]_i$ measurement in cell populations was made to resolve the regulation of EGF-induced increase of $[Ca^{2+}]_i$in human hepatoma ceils *in vitro.* Single-cell $[Ca^{2+}]_i$ was recorded continuously in fura-2-1oaded cells by using microflurometry, which made it possible to gauge with a reasonable degree of accuracy of the magnitude and the rapidity of intracellular $[Ca^{2+}]_i$ increase during EGF stimulation. The $[Ca^{2+}]_i$ was represented as the ratio of emission at 510 nm with excitations at 350 and 380 nm (350 nm/380 nm), which is a monotonic function of $[Ca^{2+}]_i$. On the other hand, the $[Ca^{2+}]_i$ determination in cell populations was performed in fluo-3-1oaded cells by using flow cytometry, which had the advantage that measurements were made on large number of single cells, and $[Ca^{2+}]_i$ was represented as the changes of the fluo-3 fluorescence intensity. As shown in Figs 1-3, EGF-induced $[Ca^{2+}]_i$ responses in BEL-7404 cells were not only dose-dependent, but also time-dependent. The data obtained by the two methods were comparable, although the falling phase of the $[Ca^{2+}]_i$ response was more sustained in suspended cells. The explanation for such discrepancy might be that the onset of $[Ca^{2+}]_i$ response to EGF stimulation is different in individual cells. Since the $[Ca^{2+}]_i$ spikes following the initial transient $[Ca^{2+}]_i$ increase were often observed in single-cell $[Ca^{2+}]_i$ analysis, an alternative explanation is that the $[Ca^{2+}]_i$ spikes in single cells may account for the sustained $[Ca^{2+}]_i$ elevation observed in cell suspension.

Although the intracellular $[Ca^{2+}]_i$ increase in hepatocytes may be brought about either as a result of mobilizing intracellular Ca^{2+} or as a result of influx of the

ion from extracellular medium[11]. The data presented in this paper demonstrated that EGF- induced $[Ca^{2+}]_i$ increase is dependent on both extracellular Ca^{2+} concentration and internal Ca^{2+} storage in endoplasmic reticulum of human hepatoma BEL-7404 cells. Chelating extracellular Ca^{2+} by excess EGTA partially inhibited the EGF-induced $[Ca^{2+}]_i$ increase, indicating that the Ca^{2+} influx occurs during EGF stimulation. There are diverse Ca^{2+} entry pathways in a variety of vertebrate cells according to the electrophysiological and biochemical criteria[12]. The nature of the stimulated Ca^{2+} entry in cultured human hepatoma BEL-7404 cells was also investigated by using Mn^{2+}, an indicator of divalent cation entry[13]. In Ca^{2+}-free assay buffer containing 50 μM Mn^{2+}, EGF induced the fluorescence quenching in fura-2-1oaded BEL-7404 cells, indicating the occurrence of bivalent cation influx during the stimulation (data not shown). Mn^{2+} possess three desirable properties. Firstly, it quenches fura-2 fluorescence so that its entry into the cytoplasma is readily detected. Secondly, Mn^{2+} entry appears to be activated under the same situation as that of Ca^{2+}, suggesting a common pathway. Thirdly, since there is no endogenous agonist-releasable Mn^{2+} store, a fluorescence quench unambiguously indicates that Mn^{2+} enters the cytoplasm from outside of the cells and not from an internal store. On the other hand, thapsigargin (TG), a tumor- promoting sesquiterpene lactone, has been proved to be able to discharge intracellular Ca^{2+} stores in rat hepatocytes by specific inhibition of endoplasmic reticulum Ca^{2+}-ATPase, as it does in many vertebrate cell types[14, 15]. Preincubation of BEL-7404 cells with 100 nM TG for 20 min, completely abolished EGF-induced $[Ca^{2+}]_i$ responses (Tab 1), which together with the observed facts of a rapid formation of inositol-1, 4, 5-trisphosphate in EGF-stimulated BEL-7404 cells (data not shown) and of the expression of antisense EGF receptor sequence in the cells suppressing the $[Ca^{2+}]_i$ response to EGF suggested that EGF stimulation linked intracellular Ca^{2+} pool was covered by TG-sensitive Ca^{2+} pool and that EGF induced Ca^{2+} release was the initial response during the stimulation. The heterologous desensitization occurred in EGF-stimulated cells pretreated with TG, but not in TG-treated cells preincubated with EGF, might reflect different mechanisms of Ca^{2+} release from ER in EGF-treatment and TG-treatment. The former was due to inositol-1, 4, 5-trisphosphate-induced Ca^{2+} release, and therefore, the depleted Ca^{2+} pool could be recovered rapidly with the rapid metabolism of inositol-1, 4, 5-trisphosphate and reuptake of Ca^{2+} by Ca^{2+}-ATPase in surface of ER. The latter was due to the net loss of Ca^{2+} from ER by the inhibition of ATP driven uptake of Ca^{2+} from cytosol and thus the exhausted Ca^{2+} pool could not be refilled rapidly. Besides the induction of Ca^{2+} discharge from endoplasmic reticulum, TG itself like EGF, triggered the Ca^{2+} influx in BEL- 7404 cells (Fig 4B), implicating that in either case, the decrease in Ca^{2+} content of the pool leads to the activation of a plasma membrane Ca^{2+} channel. Our finding, therefore, support the capacitative model proposed by Putney[16] that the depletion of inositol-1, 4, 5-trisphosphate-sensitive Ca^{2+} pool activates Ca^{2+} influx from outside of the cells. An alternative possibility is that the increase of $[Ca^{2+}]_i$, due to the discharge from ER

in either case, activates the Ca^{2+}-dependent phospholipase A_2 and then activates the Ca^{2+} channel in plasma membrane.

Taken together, EGF receptor-mediated $[Ca^{2+}]_i$ increase in the human hepatoma BEL-7404 cells are essentially dependent on the Ca^{2+} storage in endoplasmic reticulum and the internal Ca^{2+} release might be followed by the Ca^{2+} influx. The effects of the cellular Ca^{2+} movements on either cell growth and apoptosis in these cells are worth studying further.

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