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Cytosolic Ca^{2+} levels in CO25 myoblast cells bearing a human N-ras oncogene after stimulation by thapsigargin and growth factors

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Abstract: The levels of intracellular calcium [Ca^{2+}]i were examined in CO25 myoblast cells bearing a mutated human N-ras oncogene, after stimulation by thapsigargin (ThG), bombesin and epidermal growth factor (EGF). Thapsigargin induced a [Ca^{2+}]i rise mostly from intracellular calcium pools, and a small Ca^{2+} entry into cytoplasm via the plasma membrane channel. In both ras-induced and differentiated CO25 cells, [Ca^{2+}]i increase showed a similar pattern of events after addition of ThG.

The addition of EGF and bombesin to both ras-induced and differentiated cells did not induce Ca^{2+} mobilization, suggesting that the cells may not have receptors for the ligands, or desensitization of the receptors may have occured by a different signalling pathway such as stimulation of protein kinase C activity.

Key Words: N-ras, myoblast, [Ca^{2+}]i, thapsigargin, EGF, bombesin.

İnsan N-ras onkogeni taşıyan CO25 miyoblast hücrelerinde thapsigargin ve büyüme faktörleri ile stimülasyonlarından sonra hücre içi Ca^{2+} seviyeleri

Özet: CO25 miyoblast hücrelerinin, thapsigargin (ThG), bombesin ve epidermal büyüme faktörü (EGF) ile stimülasyonları sonucunda hücre içi kalsiyum [Ca^{2+}]i seviyeleri araştırılmıştır. ThG, çoğunlukla hücre içi kalsiyum depolarından salınması ve az miktarda plazma zarı kanallarından Ca^{2+} girişi yoluyla bir [Ca^{2+}]i artışına neden olmuştur. *Ras* geninin aktif olduğu yada farklılaşmış CO25 hücrelerinin her ikisinde de ThG nin hücre içi Ca^{2+} iyonlarının yükselmesi üzerine etkisi benzer bir şekilde olmuştur.

EGF ve bombesinin, *ras* geninin aktif olduğu yada farklılaşmış hücrelere eklenmesi, herhangi bir [Ca^{2+}]i hareketi oluşturmadı. Bu da hücrelerde ya bu ligandlar için reseptörler bulunmadığı yada protein kinaz C stümilasyonunun oluşturduğu gibi, farklı bir sinyal yolu tarafından reseptörlerin duyarsızlaştırılmış olabileceğini düşündürmektedir.

Anahtar Sözcükler: N-ras, miyoblast, [Ca^{2+}]i, thapsigargin, EGF, bombesin.

Introduction

Effects of *ras* oncogenes have been reported at the different levels of signal transduction pathway stimulated by growth factors. Phosphorylation of proteins on tyrosine residues by Platelet Growth Factor (PDGF) and the number of some receptors such as bradykinin, bombesin and Epidermal Growth Factor (EGF) were affected in transformed cells (1-6).

In our previous work, we have found that *N-ras* transformed CO25 cells ceased to respond to PDGF stimulation after induction of the *N-ras* gene, such as movement of Ca^{2+} from intracellular stores, phosphorylation of some proteins on tyrosine residues and expression of receptors for PDGF (6). In contrast, during differentiation of CO25 myoblasts, such failure in the responses has been not observed. It has been shown that during the transformation of skeletal muscle cells, levels of EGF, FGF and TGF-β receptors were decreased (7, 8), whereas the level of receptors for insulin and insuline like growth factors were increased (9-11).

Thapsigargin (ThG) is a naturally occurring tumor-promoting sesquiterpene lactone (12), and causes an increase in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]i$) in a variety of cell types (13-15). ThG, as a skin tumor promotor, induced the growth and blocked differentiation of mouse keratinocytes in culture, and also increased the level of $[Ca^{2+}]i$ (16). The discharge of intracellular stored Ca^{2+} by ThG occurs without hydrolysis of inositolphospholipids by specific and potent inhibition of the endoplasmic reticulum (ER) Ca^{2+} -ATPase (14, 17).

The mouse cell line (CO25) used in this study had been transfected with a glucocorticoid inducible mutated human *N-ras* oncogene under transcriptional control of the steroid-sensitive promotor of the mouse mammary tumour virus long terminal repeat (18). The cells when grown in a weakly mitogenic medium proceed to form myotubes after 4 days, and they fail to differentiate when grown in the presence of dexamethasone (DEX), displaying characteristics of a transformed phenotype (19).

Here, we examined the intracellular Ca^{2+} levels in the cells after stimulation by thapsigargin, bombesin, epidermal growth factor (EGF), bradykinin and foetal calf serum (FCS) during the transformation or the differentiation process, by spectrofluorophotometer using Fura-2 AM.

Materials and Methods

Cell culture

CO25 cells were cultured as described in Zeytinoğlu et al. (6). The cells were maintained in the growth medium (Dulbecco Modified Eagle Medium supplemented with 20% foetal calf serum (FCS), 1% L-glutamin and pencillin/streptomycin at 100 units/ml) or in the fusion promoting medium (with foetal calf serum replaced by 10% horse serum (HS)) with or without 1 μm dexamethasone (DEX). DEX was used to activate the *ras* oncogene. Cells were gassed with 10% CO2/90% air and incubated at 37°C in an incubator.

After five days in the fusion-promoting medium with or without DEX, the cells were then starved for 10-12 hours in culture medium (DMEM) in the absence of the serum to increase the sensitivity of the cells to ligands and to allow intracellular messengers to basal levels.

Fluorescence measurement of cytoplasmic free Ca^{2+}

Fura-2 AM loading

Fura-2 loading was performed by the method as in Zeytinoğlu et al. (6). Cells grown in 175 cm^2 flasks were removed and washed in DMEM (Gibco), then resuspended at a concentration of $5x10^6$ cells/ml in DMEM. The cells were loaded by the addition of the calcium indicator acetoxymethl ester of fura-2 (fura-2 AM) (Sigma) at a final concentration of 3 μM. After 30 minutes incubation at 37°C, the cells were incubated for a further 45 minutes in fresh DMEM. The cells were then washed twice in HBS (140 mM NaCl, 5 mM KCl, 1.8 mM $MgCl_2$, 10 mM glucose, 1 mM $CaCl_2$ and 10 mM Hepes, pH 7.4), and resuspended in HBS at a concentration of $2x10^6$ cells/ml. Before each experiment, the viability of cells were examined by staining with trypan blue. Generally, 80% of the cells were found to be viable.

Fluorescence measurements of cells loaded with fura-2

Fluorescence measurements were performed in a Shimadzu RF-5000 recording spectrofluorophotometer, as described in Zeytinoğlu et al. (6). For each measurement, the cell suspension was placed in a quartz cuvette and incubated at 37°C for 2-3 minutes before the addition of agonists. Determinations were performed in each experiment. Fluorescence signals were calibrated with the Kd value for Fura-2 of 226 nm.

Digitonin (BDH Lt.) was used at a concentration of 50 μg/ml, to determine maximum fluorescence of the calcium-saturated dye. To determine the Fluorescence of free dye, EGTA (Sigma), pH 9.0, was used at a concentration of 4 mM. Bradykinin, bombesin and EGF were obtained from Sigma, and used at concentrations of 1 μM, 1μM and 15 ng/ml, respectively. FCS was ontained from Gibco and used in 1% (v/v) concentration. ThG were kindly provided by Dr. A.P. Dawson (University of East Anglia) and used at a concentration of 0.1 mM. Ionomycine (a gift from Dr. G. Duncan, University of East Anglia) was used as 1 mM concentration.

Results

Thapsigargin-induced $[Ca^{2+}]i$ increase:

CO25 myoblast cells grown in three different media (FCS and HS with or without DEX), displayed a rapid, transient increase in $[Ca^{2+}]i$ upon ThG stimulation in the presence of 1 mM extracellular Ca^{2+} (Fig. 1). The ThG-induced $[Ca^{2+}]i$ rise was similar to that elicited by hormonal stimulation such as BK (Fig. 2). The $[Ca^{2+}]i$ reached a peak level immediately after addition of ThG and then slowly declined to about 70-80% of basal level within 60-80 seconds.

In the absence of extracellular Ca^{2+} by the addition of 2 mM EGTA, the addition of 0.1 mM ThG caused a similar transient increase in Ca^{2+}, but declined to about 50-60% of basal level, mostly in cells grown in HS and HS with DEX (Fig 3b and c). This results indicate that ThG mobilizes Ca^{2+} mainly from intracellular Ca^{2+} pools, and a part of Ca^{2+} enters the cytoplasm via the Ca^{2+}-ATP channels located on the plasma membrane. In cells grown in three different media, as described above, the $[Ca^{2+}]i$ increase showed a similar pattern of events after addition of ThG and FCS (Fig. 1, 2, 3 and 4).

Cytosolic Ca^{2+} levels in CO25 myoblast cells bearing a human N-ras oncogene after stimulation by thapsigargin and growth factors

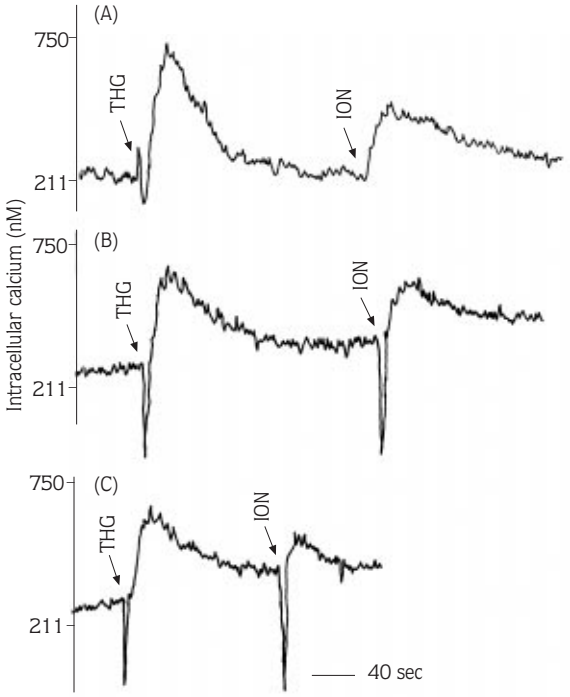

Figure 1. Effects of ThG and ionomycine (ION) on [Ca^{2+}]i in CO25 cells cultured in different media using fura-2 fluorescence dye. [Ca^{2+}]i was measured in the presence of 1 mM extracellular Ca2+ as described in Materials and Methods.
a) Cells in 20% FCS for 24 hours; b) Cells in 10% HS; or c) in 10% HS with DEX for four to five days. The times of addition of the agonists are indicated by arrows. Each plot is a representative of the plots obtained from at least three replicates of 3-5 similar experiments (separate loadings with fura-2 AM).

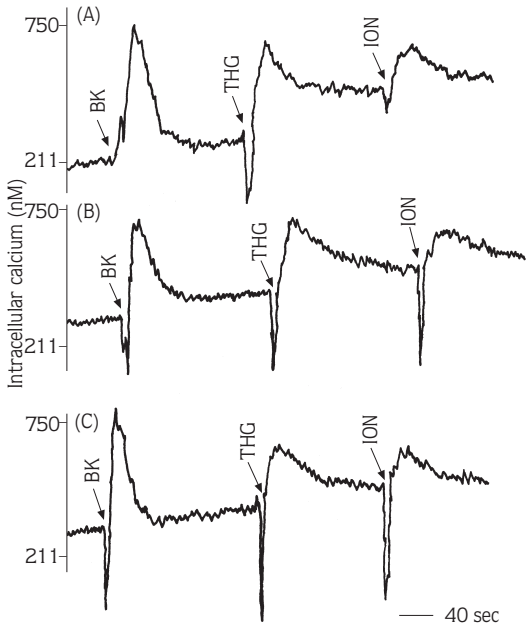

Figure 2. Effects of BK, ThG and ionomycine (ION) on [Ca^{2+}]i in CO25 cells cultured in different media using fura-2 fluorescence dye. [Ca^{2+}]i was measured in the presence of 1 mM extracellular Ca^{2+} as described in Materials and Methods.
a) Cells in 20% FCS for 24 hours; b) Cells in 10% HS; or c) in 10% HS with DEX for four to five days. The times of addition of the agonists are indicated by arrows. Each plot is a representative of the plots obtained from at least three replicates of 3-5 similar experiments (separate loadings with fura-2 AM).

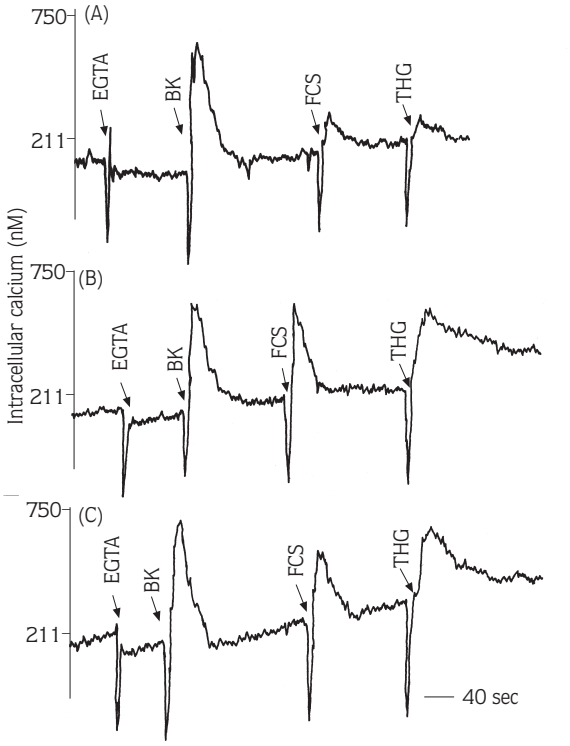

Figure 3. Effects of ThG, BK and FCS on $[Ca^{2+}]i$ in CO25 cells using fura-2 fluorescence in the absence of extracellular Ca^{2+} by addition of EGTA to the cell suspension.
a) Cells in 10% FCS for 24 hours; b) Cells in 10% HS for five days. c) in 10% HS for five days with DEX. The growth factors were employed as described in Material and Methods. The times of addition of the agonists are indicated by the arrows. Each plot is a representative of the plots obtained for at the addition of BK before ThG caused similar Ca2+ release from intracellular stores (Fig. 2c and 4c).

BK and FCS were added before the addition of ThG for comparison their effects of $[Ca^{2+}]i$ levels of cells (Fig. 2, 3 and 4). As reported previously (6), the addition of BK caused a slightly higher $[Ca^{2+}]i$ increase in ras-induced CO25 cells than in differentiated ones. Here, addition of BK before ThG caused similar Ca^{2+} release from intracellular stores (Fig. 2c and 4c).

EGF and Bombesin did not cause Ca^{2+} movement:

Addition of EGF and Bombesin to CO25 cells grown in the three different media did not induce any $[Ca^{2+}]i$ increase (Fig. 4a, b, c). Different concentrations of EGF and Bombesin were examined and found not to cause any response in these cells. However, the cells responded to the addition of other ligands such as BK, FCS and ThG after the addition of EGF and bombesin, showing the sensitivity of the cells. These results suggest that the ligands do not trigger a signaling pathway which induces a $[Ca^{2+}]i$ movement in CO25 myoblast cells, or that cells may not have receptors for these ligands.

Cytosolic Ca^{2+} levels in CO25 myoblast cells bearing a human N-ras oncogene after stimulation by thapsigargin and growth factors

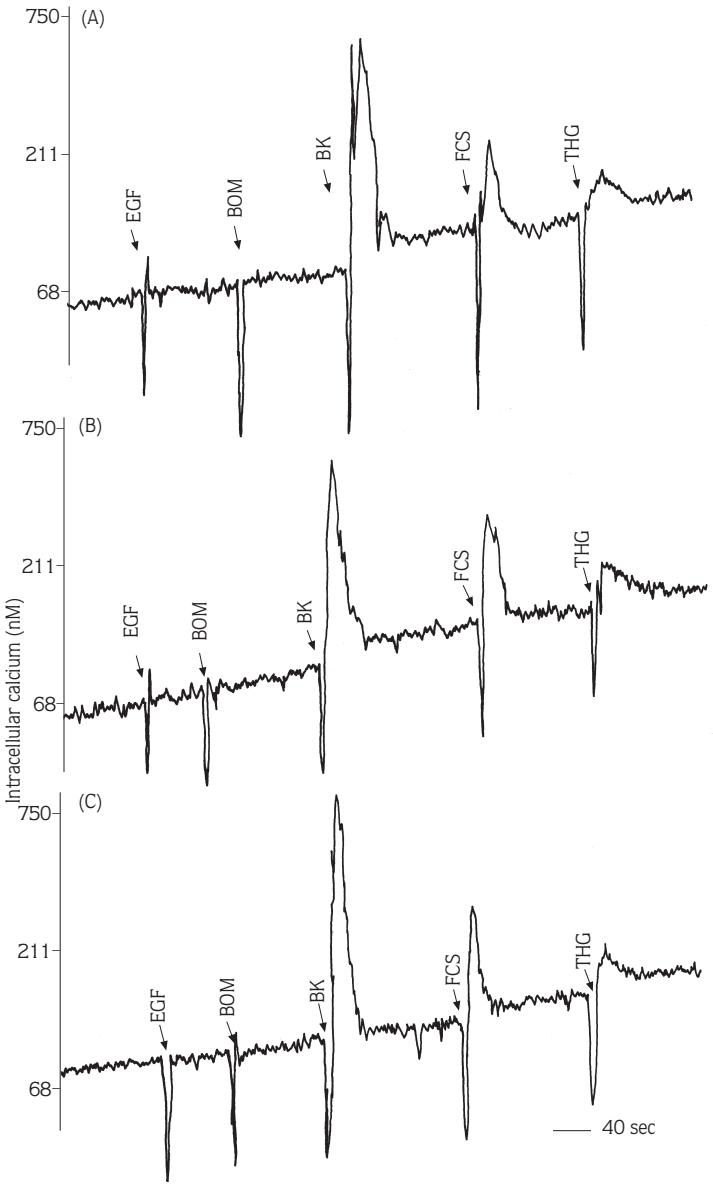

Figure 4. Effects of EGF, bombesin, BK, FCS and ThG on $[Ca^{2+}]i$ in CO25 cells cultured in different media using fura-2 fluorescence dye. $[Ca^{2+}]i$ was measured in the presence of 1 mM extracellular Ca^{2+} as described in Materials and Methods.
a) Cells in 20% FCS for 24 hours; b) Cells in 10% HS; or c) in 10% HS with DEX for four to five days. The times of addition of the agonists are indicated by the arrows. Each plot is representative of the plots obtained from at least three replicates of 3-5 similar experiments (separate loadings with fura-2 AM).

Discussion

In this study responses to ThG, EGF, bombesin and BK were examined in CO25 myoblasts bearing a steroid-inducible mutated N-ras oncogene grown in the growth medium (FCS) and differentiation medium (HS) with or without DEX.

Thapsigargin has been used as a probe for intracellular Ca^{2+} storage and release processes in various cell systems (13-15, 17, 20-24). Generally, ThG causes a $[Ca^{2+}]i$ rise slower than that elicited by hormonal stimulation, not concomitant breakdown of inositolphospholipids with the production inositol phosphates, and an initiation of $[Ca^{2+}]i$ rise by release of intracellular sequestered Ca^{2+}. It has also been shown that ThG discharges stored Ca^{2+} from the ER by a direct interaction (17). However, in a few examples, such as a neuroblastoma cell line NG115-401L, ThG releases intracellular Ca^{2+} without an accompanying influx, suggesting that receptor-coupled production of a code signal may be required (13).

In this study, we have shown that ThG induces a large increase in $[Ca^{2+}]i$ in both ras-induced and differentiated CO25 myoblasts (Fig. 1). The ThG-induced increase in $[Ca^{2+}]i$ is attributed to Ca^{2+} mobilization from intracellular Ca^{2+} pools, because ThG causes a transietn $[Ca^{2+}]i$ response in the absence of extracellular Ca^{2+} after the addition of EGTA (Fig. 3). In the presence of 1mM of extracellular Ca^{2+}, $[Ca^{2+}]i$ decreased to 60-70% of the basal level. The little sustained elevation of $[Ca^{2+}]i$ indicates that ThG activates to enter some part of Ca^{2+} across the plasma membrane. This result is consistent with the findings of Fujiki et al. (20) and Jackson et al. (13), whereas it is in conflict with the findings of others (14, 15, 21-24).

BK and FCS caused an increase in $[Ca^{2+}]i$ before and after the addition of ThG in a pattern similar to that reported previously (6), indicating that Ca^{2+} is released from intracellular stores (Fig. 2, 3, 4).

Intracellular free calcium has a critical role in the regulation of cell growth by inducing mitogenesis in Growth factor-stimulated cells such as PDGF (25, 26). In a previous study (6), we showed that during differentiation of CO25 cells, the increase in $[Ca^{2+}]i$ stimulated by PDGF-BB or BK was diminished, and during transformation, the response to PDGF-BB was completely lost. Bombesin appears to have biochemical and physical properties similar to the peptide BK, which has been shown to act as a mitogen and in a paracrine fashion as a local hormone (27).

Since certain other growth factor receptors, such as EGF, FGF and TGF-beta, have been shown to be deactivated or to decrease in number upon differentiation (7, 8, 28), and to increase in many human tumor cells (29), we investigated the effects of EGF and Bombesin on $[Ca^{2+}]i$ mobilization in both ras-induced and differentiated CO25 cells. In ras-transformed fibroblasts, Ca^{2+} release from internal stores by bombesin stimulation was completely inhibited and the number of bombesin receptors was decreased (3).

Here, the addition of different concentrations of EGF and bombesin did not cause $[Ca^{2+}]i$ mobilization in either ras-induced or differentiated CO25 cells. In A-413 and 3T3 cells, EGF evoked a Ca^{2+} signal by the activation of a Ca^{2+} influx pathway in the plasma membrane (30). However, activated protein kinase C catalyzes phosphorylation of the EGF receptor at threonine 654, resulting in decreased affinity of the receptor for EGF, decreased EGF-stimulated protein

tyrosine kinase activity and inhibition of ligand-induced receptor internalization (31).

In CO25 myoblast cells, EGF and bombesin may act through their receptors by activating a signalling pathway which does not affect $[Ca^{2+}]i$ mobilization, or cells may not have receptors for the ligands. Perhaps an activation of protein kinase C may decrease the affinity of the receptors. All of these possibilities remain to be tested.

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