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# Caffeine induced $Ca^{2+}$ release and capacitative $Ca^{2+}$ entry in human embryonic kidney (HEK293) cells

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**Abstract**

The potential role of endogenous ryanodine receptor (RyR) in modulating $Ca^{2+}$ handling in HEK293 cells is controversial. Using Fura2/AM, here we provide evidence that caffeine can induce $Ca^{2+}$ release from inositol 1,4,5-trisphosphate receptor-sensitive stores and $Ca^{2+}$ entry in early passage numbers of HEK293 cells, but not in late passage ones. Ryanodine blocks caffeine-mediated effect, whereas 4-chloro-*m*-cresol can mimic these effects. In contrast, an increase in cyclic AMP or activation of voltage-dependent $Ca^{2+}$ channels does not induce detectable alteration in intracellular $Ca^{2+}$. Importantly, immunoblotting and staining have revealed that endogenous RyR expression is more abundant in the early than in the late passage cells. Additionally, similar to carbachol, $Ca^{2+}$ entry in response to caffeine is blocked by capacitative $Ca^{2+}$ entry inhibitors. These results indicate that the endogenous RyR in HEK293 cells can function as $Ca^{2+}$ release channels and mediate capacitative $Ca^{2+}$ entry, but they may be reduced due to cell passage.


*Keywords:* Ryanodine receptor; HEK293 cells; $Ca^{2+}$ release; Capacitative $Ca^{2+}$ entry

## 1. Introduction

Elevating intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) is mainly via $Ca^{2+}$ release from internal $Ca^{2+}$ stores or/and $Ca^{2+}$ entry across plasma membrane, and both processes are coupled with and controlled by a variety of cellular signaling pathways (Berridge et al., 2000; Putney and Ribeiro, 2000; Kiselyov et al., 2003). $Ca^{2+}$ signaling in non-excitable cells has traditionally been ascribed to agonist-induced release of $Ca^{2+}$ from intracellular stores, which bear the ubiquitous inositol 1,4,5-trisphosphate-gated $Ca^{2+}$ channel/receptor ($IP_3R$), and subsequent $Ca^{2+}$ entry. This particular $Ca^{2+}$ influx in such context is termed capacitative $Ca^{2+}$ entry (Putney, 1986; Venkatachalam et al., 2002). Recently, gene expression of ryanodine receptor (RyR), another class of intracellular releasing $Ca^{2+}$ channels, which predominates in excitable cells, has been detected in a number of non-excitable cells and tissues, and functional $Ca^{2+}$ release associated with the receptor has been observed in some of the cell types (Leite et al., 1999; Ricard et al., 1997; Xue et al., 2000; Querfurth et al., 1998; Tunwell and Lai, 1996). However, it remains controversial as to whether endogenous RyR regulates $[Ca^{2+}]_i$ in non-excitable cells, including HEK293 cells (Bennett et al., 1996; Rossi et al., 2002; Tong et al., 1999).

HEK293 cell line has been widely used for functional and biochemical studies on ionic channel proteins. Recently, it has been utilized to express RyR (including all three isoforms) for structure–function studies of the receptor (Rossi et al., 2002; Xiao et al., 2002). It is, therefore, important to determine whether endogenous functional RyR is present in HEK293 cells. In the present study, we compared the effects of caffeine and carbachol, two known modulators respectively for RyR and $IP_3R$ on $Ca^{2+}$ amplitude and kinetics (Pessah et al., 1987; Luo et al.,

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2001), and found that endogenous RyR plays an important role in regulating intracellular $Ca^{2+}$ mobilization, and that this function is significantly reduced in late passage cells.

## 2. Materials and methods

### 2.1. Cell culture

HEK293 cells obtained from the ATCC were cultured at 37 °C in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum and 2 mM glutamine in a humidified 95% air, 5% $CO_2$ incubator. Two different batches of cells were used in the present study. Experiments with the early batch of HEK293 cells were performed between passage numbers P6 to P15, whereas the late batch was between passage numbers P25 to P35. For $Ca^{2+}$ measurements, cells were plated on glass coverslips or 35 mm glass bottom microwell dishes (MatTek Co. USA), cultured to about 70% confluence, and used within 24–30 h after plating.

### 2.2. $Ca^{2+}$ fluorescence measurements

Fluorescence measurements in groups of HEK293 cells were described previously (Luo et al., 2001). Briefly, the coverslips with attached cells were mounted in a Teflon chamber and incubated with 1 μM Fura 2/AM (Molecular Probes) in serum-free DMEM at 37 °C for 25 min. Then the cells were washed three times and incubated for 30 min at room temperature in HEPES-buffered physiological saline solution (HPSS in mM: NaCl 120, KCl 5.4, $Mg_2SO_4$ 0.8, HEPES 20, $CaCl_2$ 1.8, Glucose 10, pH 7.4 adjusted by NaOH). $Ca^{2+}$-free solution contained no added $CaCl_2$ in HPSS. All the $Ca^{2+}$ measurements were performed at room temperature (22±1 °C) and carried out within 2 h of loading for each coverslip.

To obtain instant exposure to caffeine stimulation, we employed stop flow method for all medium changes in the whole study. In this method, we changed medium by aspirating the remained fluid and replacing bathing solution within 5 s.

### 2.3. Immunoprecipitation and Western blotting

Both batches of HEK293 cells, neonatal rat ventricular myocytes (NRVMs, as a positive control) and fibroblasts (as a negative control) were lysed in RIPA buffer (Santa Cruz) containing 1 mM polymethylsulfonyl fluoride (PMSF), and 2 μg/ml protease inhibitor cocktail (Santa Cruz) for 1 h on ice. Homogenates were sonicated on ice for three bursts of 5 s each and centrifuged for 15 min at 14,000×*g* at 4 °C. For immunoprecipitations, 200 μg NRVM and 400 μg HEK293 cell and fibroblast lysates were incubated with 5 μl of mouse IgG1 and 30 μl protein G plus-Agarose (Santa Cruz) for 1 h on ice with slight agitation to eliminate nonspecific binding. The supernatant was incubated at 4 °C for 1 h under continuous shaking with anti-RyR type 1 and 2 antibody (1:500, ABR), and adsorbed onto 30 μl protein G plus-Agarose overnight at 4 °C, according to manufacturer instructions (Santa Cruz). At the end of the incubation, complexes immunoadsorbed to the beads were washed three times with RIPA buffer, and the supernatant was diluted in sample buffer to be analyzed for RyR expression by Western blotting as described below.

The immunoprecipitates and 50 μg lysates in each type of cells were heated for 5 min and resolved on a 3–8% SDS–PAGE gel (Invitrogen), transferred to nitrocellulose, and immunoblotted with two mouse anti-RyR antibodies; one strongly binds with type 2 RyR and another detects both type 1 and 2 RyR (ABR), at dilution of 1:1000 or 1:3000, respectively. The primary antibody was then removed, and the membrane was incubated with horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected by using enhanced chemiluminescence.

### 2.4. Fluorescence staining

Cultured HEK293 cells, NRVMs and fibroblasts were washed with HPSS, fixed with 4% formaldehyde (Sigma) and then permeabilizied with 0.1% Triton X-100. The anti-RyR type 1 and 2 antibody was used at dilution of 1:800. Secondary antibody was anti-mouse CY™ 5 (Vector Lab, CA) at a dilution of 1:1000. Chemifluorescent detection was directly performed on a laser scanning confocal microscopy (Zeiss) with an X40 oil-immersion objective (NA 1.3).

### 2.5. Statistics

For some experiments, the average peak responses (the ratio of Fura2 fluorescence due to excitation at 340 nm to that excited by at 380 nm light) were calculated and expressed as means±S.E.M. for the indicated number ($n$) of experiments. Statistical significance was determined with the unpaired Student's $t$ test ($p<0.05$).

### 2.6. Materials

Carbachol, 4-chloro-*m*-cresol and 2-aminoethyoxydiphenyl borane were from Calbiochem (La Jolla, CA, USA). Caffeine, gadolinium, ryanodine and all the other reagents were obtained from Sigma (St. Louis, MO, USA).

## 3. Results

### 3.1. Caffeine induces $Ca^{2+}$ signaling via activation of ryanodine receptor in early batch HEK293 cells

Two batches of HEK293 cells were divided according to their passage numbers (see Materials and Methods). Treatment of the early batch cells with caffeine caused a

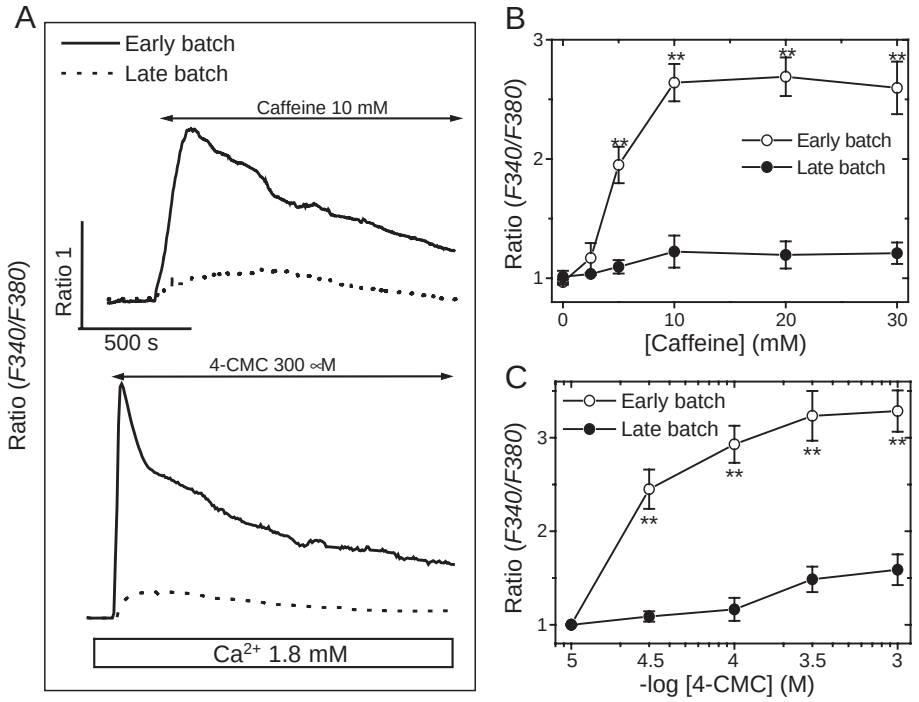


Fig. 1. Ryanodine receptor agonists induce intracellular $Ca^{2+}$ increase in early passage HEK293 cells. Two batches of Fura 2/AM loaded HEK293 cells were divided (see Materials and Methods) and activated with RyR agonists in $Ca^{2+}$ containing medium. Panel *A* shows representative examples of $[Ca^{2+}]_i$ response to either 10 mM caffeine (top panel) or 300 μM 4-CMC treatment (bottom panel) in early batch (*solid traces*) and late batch (*dotted traces*) cells. Panels *B* and *C* illustrate their concentration-dependent responses in both batches of cells, respectively (as indicated). Each point represents means±S.E.M. from 4 to 6 experiments. ** stands for $P<0.01$ vs. late batch cells.

concentration-dependent increase in $[Ca^{2+}]_i$ in $Ca^{2+}$-containing HPSS. Interestingly, it had a very minor effect in late batch cells (Fig. 1A and B), while carbachol and thapsigargin retained their effectiveness on these cells (data not shown). This indicates that passaging cells to late passage numbers specifically influences $[Ca^{2+}]_i$ response to caffeine. The RyR-mediated $[Ca^{2+}]_i$ increase was also confirmed by using 4-chloro-*m*-cresol (4-CMC), a potent and specific activator of the skeletal muscle RyR (Herrmann-Frank et al., 1996). As shown in Fig. 1A and C, it dramatically increased $[Ca^{2+}]_i$ in a concentration-dependent manner in the early, but not in the late batch of HEK293 cells.

To identify the $Ca^{2+}$ signaling pathway responding to caffeine, we stimulated early batch cells with 10 mM caffeine in nominally $Ca^{2+}$-free HPSS. Like the effect of 100 μM carbachol, caffeine induced a transient rise of $[Ca^{2+}]_i$ followed by a more sustained entry of $Ca^{2+}$ when $Ca^{2+}$ was added back to the medium (Fig. 2A), indicating two components in $[Ca^{2+}]_i$ increase: a release of $Ca^{2+}$ from internal sites and $Ca^{2+}$ entry from extracellular milieu.

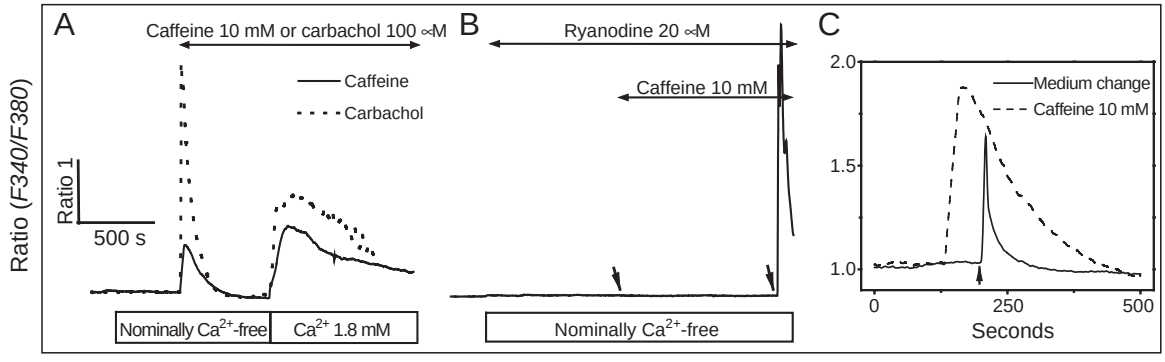


Fig. 2. Caffeine-induced $Ca^{2+}$ release is mediated by activation of ryanodine receptor. Panel *A*, $Ca^{2+}$ was initially absent from the medium and was restored to 1.8 mM were indicated. Similar as 100 μM carbachol (*dotted trace*), 10 mM caffeine (*solid trace*) induced transient $Ca^{2+}$ release followed by a $Ca^{2+}$ entry phase upon restoration of $Ca^{2+}$. Panel *B*, in nominally $Ca^{2+}$-free medium, pretreatment of cells with 20 μM ryanodine for 10 min blocked $Ca^{2+}$ release induced by caffeine, but not that induced by carbachol (indicated with the second arrow). Panel *C*, a typical $[Ca^{2+}]_i$ transient was caused by medium replacement (indicated by an arrow). Similar results to each curve depicted were obtained in a total of 5 to 7 experiments.

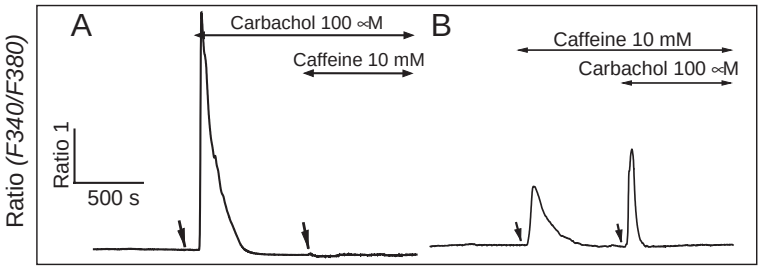


Fig. 3. Caffeine-induced mobilization of $Ca^{2+}$ is originated from inositol 1,4,5-trisphsphate-sensitive $Ca^{2+}$ pool in HEK293 cells. Panels *A* and *B*, in nominally $Ca^{2+}$-free medium, cells were treated with either 100 μM carbachol or 10 mM caffeine (first arrows). Subsequently the caffeine-treated cells were exposed to carbachol, and the carbachol-treated cells were stimulated with caffeine (second arrows). These results are representatives of a total of 4 to 6 similar experiments.

Pretreatment of cells with 20 μM ryanodine for 10 min, a selective RyR inhibitor (Johnson et al., 2004), completely blocked caffeine-induced $Ca^{2+}$ release, but not carbachol effect (Fig. 2B). A similar result was also observed in 4-CMC treated cells (data not shown).

In addition to activating RyR, caffeine is also supposed to accumulate cyclic AMP via inhibition of phosphodiesterase, and cyclic AMP may affect $Ca^{2+}$ release or/and $Ca^{2+}$ influx in various cell types (Cooper et al., 1995; Nishiyama et al., 2003). Thus, we employed 10 μM forskolin, an adenylcyclase activator to increase cyclic AMP or 50 mM KCl to examine the possible involvement of cyclic AMP and voltage-dependent $Ca^{2+}$ channels in the regulation of $[Ca^{2+}]_i$, and found that they had no effect on $[Ca^{2+}]_i$ in $Ca^{2+}$-containing medium (data not shown).

Additionally, consistent with early report (Tong et al., 1999), a transient $[Ca^{2+}]_i$ increase could also be observed immediately after medium change in some of the experiments (Fig. 2C, 7 cases out of 58 medium replacements). However, the duration of this $[Ca^{2+}]_i$ transient was much shorter than that of caffeine-induced $[Ca^{2+}]_i$ signaling. Therefore, caffeine-mediated $[Ca^{2+}]_i$ response is specific and likely due to activation of endogenous RyR in early batch HEK293 cells.

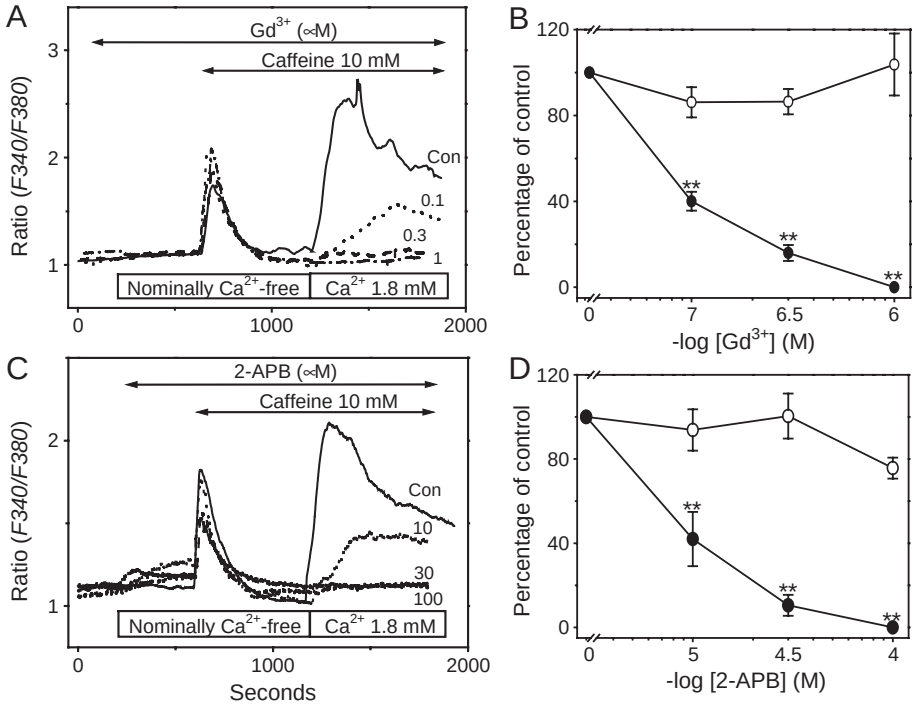


Fig. 4. Effects of $Gd^{3+}$ and 2-APB on caffeine-induced $Ca^{2+}$ release and $Ca^{2+}$ entry in HEK293 cells. The protocols in Panels *A* and *C* were identical to that in Fig. 2A, except for pretreatments of some experiments with antagonists. In some traces (as indicated), each concentration of $Gd^{3+}$ (μM) in *A* or 2-APB (μM) in *C* was added to the medium when $Ca^{2+}$ was removed and was present throughout the re-addition of $Ca^{2+}$. Panels *B* and *D* illustrate summary data of $Gd^{3+}$ (*B*) and 2-APB (*D*) inhibitory effects on $Ca^{2+}$ release (*open circles*) and $Ca^{2+}$ entry (*filled circles*) phases of the response to 10 mM caffeine. Each point represents means±S.E.M. from 5 to 6 experiments. ** stands for $P<0.01$ compared between control and antagonist treated group.

### 3.2. Caffeine-induced $Ca^{2+}$ release from $IP_3$-sensitive store and capacitative $Ca^{2+}$ entry

To determine the characteristic features of caffeine-mediated $Ca^{2+}$ signaling, we next challenged cells with 10 mM caffeine and 100 μM carbachol sequentially, or vice versa in nominally $Ca^{2+}$-free medium. As Fig. 3A shows, no further $Ca^{2+}$ discharge was observed when 10 mM caffeine added to carbachol-pretreated cells, while addition of 100 μM carbachol subsequent to caffeine induced a moderate additional $Ca^{2+}$ release, indicating that caffeine and carbachol mobilize the same or related $Ca^{2+}$ pool in this cell type.

Depletion of internal $Ca^{2+}$ stores presumably results in activation of capacitative $Ca^{2+}$ entry in non-excitable cells (Putney, 1986; Venkatachalam et al., 2002; Berridge et al., 2000). Thus, we further clarify whether the $Ca^{2+}$ entry due to caffeine is also capacitative by using gadolinium ($Gd^{3+}$), at concentration ≤l μM, and 2-aminoethyoxydiphenyl borane (2-APB), two potent inhibitors of capacitative $Ca^{2+}$ entry (Luo et al., 2001; Bootman et al., 2002). Equal potently, the two reagents concentration-dependently inhibited caffeine-induced $Ca^{2+}$ influx, whereas the $Ca^{2+}$ release induced by caffeine was not affected (Fig. 4). These results clearly demonstrate that caffeine discharges $IP_3$-sensitive $Ca^{2+}$ store and subsequently activates capacitative $Ca^{2+}$ entry in HEK293 cells.

### 3.3. Detection of endogenous RyR in HEK293 cells

The above results suggest that endogenous RyR may be expressed in HEK293 cells. Thus, we identified the endogenous RyR expression with anti-RyR type 2 and also anti-RyR type 1 and 2 antibodies, respectively, because of no commercially available anti-RyR type 1 antibody. Cardiomyocytes and cardiac fibroblasts were used as a positive and a negative control (Bennett et al., 1996), respectively. Using conventional Western blot, we failed to detect obvious immunoreaction band in either batch of HEK293 cells using either antibody, unlike in cardiomyocytes. But using immunoprecipitation and Western blot combination, a positive band was found in 560 kDa in the early batch of HEK293 using anti-RyR type 1 and 2 antibody, but not when anti-RyR type 2 antibody is used. Cardiomyocytes, however, showed positive bands in blotting with both antibodies (Fig. 5A and B), suggesting that RyR type 1 may be expressed in the early batch of HEK293 cells. Further immunostaining revealed a cytoplasmic distribution of the signal (Fig. 5C and E). The late batch of cells, however, showed no obvious labeling in immunoblotting (Fig. 5D), although positive, but weak, staining was found compared with the early HEK293 cells and fibroblasts (Fig. 5F). These results suggest that a decrease in the endogenous RyR expression may occur after cells culturing to late passages.

## 4. Discussion

The most important finding of the present study is the demonstration of functional RyR in HEK293 cells. This conclusion is based on following lines of evidence. We, first, established that HEK293 cells possess caffeine-sensitive $Ca^{2+}$ release as well as $Ca^{2+}$ influx (Figs. 1 and

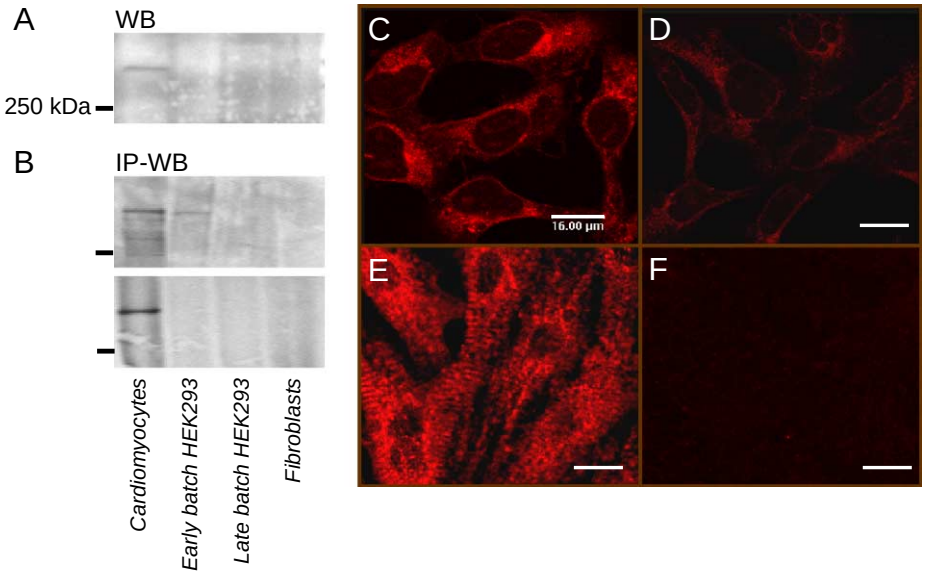


Fig. 5. Endogenous ryanodine receptor expression in HEK293 cells. Panel *A* shows immunoblotting (WB) of whole cell lysate proteins from cultured neonatal rat cardiomyocytes, fibroblasts and two batches of HEK293 cells using an anti-RyR type 2 antibody. Panel *B* presents immunoblotting (IP-WB) of the immunoprecipitates from the four different cell culture lysates using an anti-RyR for both type 1 and 2 antibody (upper panel) and an anti-RyR for type 2 antibody (bottom panel). Panels *C–F* illustrate immuno-labeling and localization of RyR in cultured early batch HEK293 cells (*C*), late batch HEK293 cells (*D*), neonatal rat cardiomyocytes (*E*) and fibroblasts ( *F*) using an anti-RyR type 1 and 2 antibody. The scale bars at the bottom right of each panel represent 16 μm. These results are representatives of a total of 4 experiments.

2). This transient $Ca^{2+}$ increase, about 1/3 of that induced by 100 μM carbachol, was completely abrogated by pre-discharge of $Ca^{2+}$ pool with carbachol (Fig. 3), indicating an overlap with $IP_3$-sensitive $Ca^{2+}$ store. Similar to those of store-depleting agents, the extracellular $Ca^{2+}$-dependent $Ca^{2+}$ elevation induced by caffeine was sensitive to both 2-APB and $Gd^{3+}$ inhibition in a concentration-dependent manner (Fig. 4, and also see Luo et al., 2001). 2-APB has currently been documented to be efficient and specific in preventing capacitative $Ca^{2+}$ entry (Bootman et al., 2002). $Gd^{3+}$ at concentrations ≤1 μM is shown to abolish capacitative $Ca^{2+}$ entry in HEK293 cells (Mignen et al., 2003; Luo et al., 2001). Therefore, the $Ca^{2+}$ influx in response to caffeine is likely due to an activation of capacitative $Ca^{2+}$ entry as a consequence of caffeine-mediated depletion of $IP_3$-sensitive $Ca^{2+}$ store.

Second and perhaps more importantly, the signaling pathway for caffeine-mediated $Ca^{2+}$ release is likely via activation of endogenous RyR, because of complete abolishment of the caffeine effect by ryanodine (Fig. 2B) and the failure of increased cAMP or activator of voltage-dependent $Ca^{2+}$ channel to elevate $[Ca^{2+}]_i$. Furthermore, expression of endogenous RyR was detected in immunoblotting and staining (Fig. 5), consistent with the previous notion of Querfurth et al. (1998). However, controversial data have also been reported in the same cell line and this discrepancy was interpreted by different methods used, i.e. perfusion vs. 'stop flow' method (Tong et al., 1999). In this study a transient $[Ca^{2+}]_i$ increase due to medium change by "stop flow" method was observed. However, compared with the responses to caffeine, this $[Ca^{2+}]_i$ rise occurred occasionally with much smaller amplitude and duration (Fig. 2C). Additionally, a much larger and prolonged $Ca^{2+}$ entry was followed by readdition of $Ca^{2+}$ in caffeine-stimulated cells (Fig. 2A), but not in medium change. Thus, it is unlikely that $[Ca^{2+}]_i$ elevation induced by medium change accounts for the caffeine effects manifested in this study.

Recently, endogenous RyR, the predominant $Ca^{2+}$ release receptor/channels expressed in excitable cells, has been found in several non-excitable cells, whereas inconsistent responses to caffeine or ryanodine have been reported in some cell lines such as Jurkat T and Hela cells (Ricard et al., 1997; Bennett et al., 1996; Missiaen et al., 1994; Hosoi et al., 2001). Interestingly, in this study we have demonstrated that the number of HEK293 cell passages determines whether the cell is able to respond to caffeine and exhibits endogenous RyR expression. HEK293 cells during early passage numbers consistently respond to activators of RyR, but this ability is lacking when they grow to late passages (Fig. 1), while their responses to carbachol is unchanged. Since caffeine and carbachol may share the same internal $Ca^{2+}$ store (Fig. 3), the different response to RyR activators is unlikely due to high cell passage-associated alterations in $Ca^{2+}$ signaling. Alternatively, the endogenous RyR expression is significantly reduced when cells come to late passage numbers, as evidenced by decreased intensity in immunostaining and lack of immunoreactive band in Western blot with RyR specific antibodies (Fig. 5). The mechanism underlying the cell passage-related decrease in RyR expression merits further investigation. Similarly, studies in Jurkat T cells and mink lung epithelial cells suggest that the endogenous RyR expression is related with the state of cell growth; cells in high growth state express more functional RyR than those in low growth stage (Hosoi et al., 2001; Giannini et al., 1992).

In summary, this study provides evidence for the existence of functional endogenous RyR in HEK293 cells. Activation of this receptor leads to both $Ca^{2+}$ release and capacitative $Ca^{2+}$ entry. However, prolongation of the cell passage markedly reduces the receptor expression as well as its function. Thus, our study might provide the interpretation for the inconsistent previous reports regarding the existence and functionality of endogenous RyR in HEK293 cells, since different cell preparations might be used in different studies.

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## References

Bennett, D.L., Cheek, T.R., Berridge, M.J., Smedt, H.D., Parys, J.B., Missiaen, L., Bootman, M.D., 1996. Expression and function of ryanodine receptors in nonexcitable cells. J. Biol. Chem. 271, 6356–6362.

Berridge, M.J., Lipp, P., Bootman, M.D., 2000. The versatility and universality of calcium signalling. Nat. Rev., Mol. Cell Biol. 1, 11–21.

Bootman, M.D., Collins, T.J., Mackenzie, L., Roderick, H.L., Berridge, M.J., Peppiatt, C.M., 2002. 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated $Ca^{2+}$ entry but an inconsistent inhibitor of $InsP_3$-induced $Ca^{2+}$ release. FASEB J. 16, 1145–1150.

Cooper, D., Mons, N., Karpen, J., 1995. Adenylyl cyclases and the interaction between calcium and cAMP signaling. Nature 374, 421–424.

Giannini, G., Clementi, E., Ceci, R., Marziali, G., Sorrentino, V., 1992. Expression of a ryanodine receptor-$Ca^{2+}$ channel that is regulated by TGF-beta. Science 257, 91–94.

Herrmann-Frank, A., Richter, M., Sarkozi, S., Mohr, U., Lehmann-Horn, F., 1996. 4-Chloro-*m*-cresol, a potent and specific activator of the skeletal muscle ryanodine receptor. Biochim. Biophys. Acta 1289, 31–40.

Hosoi, E., Nishizaki, C., Gallagher, K.L., Wyre, H.W., Matsuo, Y., Sei, Y., 2001. Expression of the ryanodine receptor isoforms in immune cells. J. Immunol. 167, 4887–4894.

Johnson, J.D., Kuang, S., Misler, S., Polonsky, K.S., 2004. Ryanodine receptors in human pancreatic beta cells: localization and effects on insulin secretion. FASEB J. 18, 878–880.

Kiselyov, K., Shin, D.M., Muallem, S., 2003. Signalling specificity in GPCR-dependent $Ca^{2+}$ signaling. Cell. Signal. 15, 243–253.

Leite, M.F., Dranoff, J.A., Gao, L., Nathanson, M.H., 1999. Expression and subcellular localization of the ryanodine receptor in rat pancreatic acinar cells. Biochem. J. 337, 305–309.

Luo, D., Broad, L.M., Bird, G.J., Putney Jr., J.W., 2001. Signaling pathway underlying muscarinic receptor-induced $[Ca^{2+}]_i$ oscillation in HEK293 cells. J. Biol. Chem. 276, 5613–5621.

Mignen, O., Thompson, J.L., Shuttleworth, T.J., 2003. $Ca^{2+}$ selectivity and fatty acid specificity of the noncapacitative, arachidonate-regulated $Ca^{2+}$ (ARC) channels. J. Biol. Chem. 278, 10174–10181.

Missiaen, L., De Smedt, H., Pary, J.B., Oike, M., Casteels, R., 1994. Kinetics of empty store-activated $Ca^{2+}$ influx in HeLa cells. J. Biol. Chem. 269, 5817–5823.

Nishiyama, M., Hoshino, A., Tsai, L., Henley, J.R., Goshima, Y., Tessier-Lavigne, M., Poo, M.M., Hong, K., 2003. Cyclic AMP/GMP-dependent modulation of $Ca^{2+}$ channels sets the polarity of nerve growth-cone turning. Nature 424, 990–995.

Pessah, I.N., Stambuk, R.A., Casida, J.E., 1987. $Ca^{2+}$-activated ryanodine binding: mechanisms of sensitivity and intensity modulation by $Mg^{2+}$, caffeine, and adenine nucleotides. Mol. Pharmacol. 31, 232–238.

Putney Jr., J.W., 1986. A model for receptor-regulated calcium entry. Cell Calcium 7, 1–12.

Putney Jr., J.W., Ribeiro, C.M., 2000. Signaling pathways between the plasma membrane and endoplasmic reticulum calcium stores. Cell. Mol. Life Sci. 57, 1272–1286.

Querfurth, H.W., Haughey, N.J., Greenway, S.C., Yacono, P.W., Golan, D.E., Geiger, J.D., 1998. Expression of ryanodine receptors in human embryonic kidney (HEK293) cells. Biochem. J. 334, 79–86.

Ricard, I., Martel, J., Dupuis, L., Dupuis, G., Payet, M.D., 1997. A caffeine/ryanodine-sensitive $Ca^{2+}$ pool is involved in triggering spontaneous variations of $Ca^{2+}$ in Jurkat T lymphocytes by a $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) mechanism. Cell. Signal. 9, 197–206.

Rossi, D., Simeoni, I., Micheli, M., Bootman, M., Lipp, P., Allen, P.D., Sorrentino, V., 2002. RyR1 and RyR3 isoforms provide distinct intracellular $Ca^{2+}$ signals in HEK 293 cells. J. Cell Sci. 115, 2497–2504.

Tong, J., Du, G.G., Chen, S.R.W., Maclennan, D.H., 1999. HEK-293 cells possess a carbachol- and thapsigargin-sensitive intracellular $Ca^{2+}$ store that is responsive to stop-flow medium changes and insensitive to caffeine and ryanodine. Biochem. J. 343, 39–44.

Tunwell, R.E.A., Lai, F.A., 1996. Ryanodine receptor expression in the kidney and a non-excitable kidney epithelial cell. J. Biol. Chem. 271, 29583–29588.

Venkatachalam, K., van Rossum, D.B., Patterson, R.L., Ma, H.T., Gill, D.L., 2002. The cellular and molecular basis of store-operated calcium entry. Nat. Rev., Mol. Cell Biol. 4, 263–272.

Xiao, B., Masumiya, H., Jiang, D., Wang, R., Sei, Y., Zhang, L., Murayama, T., Ogawa, Y., Lai, F.A., Wagenknecht, T., Chen, S.R., 2002. Isoform-dependent formation of heteromeric $Ca^{2+}$ release channels (ryanodine receptors). J. Biol. Chem. 277, 41778–41785.

Xue, H.H., Zhao, D.M., Suda, T., Uchida, C., Oda, T., Chida, K., Ichiyama, A., Nakamura, H., 2000. Store depletion by caffeine/ryanodine activates capacitative $Ca^{2+}$ entry in nonexcitable A549 cells. J. Biochem. (Tokyo) 128, 329–336.