Effect of m-3M3FBS on Ca\textsuperscript{2+} Movement in PC3 Human Prostate Cancer Cells

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Abstract

The effect of 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (m-3M3FBS), a presumed phospholipase C activator, on cytosolic free Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}) in PC3 human prostate cancer cells is unclear. This study explored whether m-3M3FBS changed basal [Ca\textsuperscript{2+}]\textsubscript{i} levels in suspended PC3 cells by using fura-2 as a Ca\textsuperscript{2+}-sensitive fluorescent dye. M-3M3FBS at concentrations between 10-50 \(\mu\text{M}\) increased [Ca\textsuperscript{2+}]\textsubscript{i} in a concentration-dependent manner. The Ca\textsuperscript{2+} signal was reduced by 60\% by removing extracellular Ca\textsuperscript{2+}. M-3M3FBS-induced Ca\textsuperscript{2+} influx was inhibited by the store-operated Ca\textsuperscript{2+} channel blockers nifedipine, econazole and SK&F96365, and by the phospholipase A2 inhibitor aristolochic acid. In Ca\textsuperscript{2+}-free medium, 30 \(\mu\text{M}\) m-3M3FBS pretreatment greatly inhibited the [Ca\textsuperscript{2+}]\textsubscript{i} rise induced by the endoplasmic reticulum Ca\textsuperscript{2+} pump inhibitor thapsigargin or BHQ. Conversely, pretreatment with thapsigargin, BHQ or cyclopiazonic acid reduced the major part of m-3M3FBS-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise. Inhibition of phospholipase C with U73122 did not much alter m-3M3FBS-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise. Collectively, in PC3 cells, m-3M3FBS induced [Ca\textsuperscript{2+}]\textsubscript{i} rises by causing phospholipase C-independent Ca\textsuperscript{2+} release from the endoplasmic reticulum and Ca\textsuperscript{2+} influx via store-operated Ca\textsuperscript{2+} channels.

Key Words: Ca\textsuperscript{2+}, m-3M3FBS, PC3, prostate

Introduction

Bae \textit{et al}. (1) has reported a compound: 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (m-3M3FBS) that was thought to evoke a transient cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) increase in neutrophils by stimulation of phospholipase C (PLC). Subsequent studies have used m-3MFBS as a selective PLC activator in various systems, including snail neurons (27), membrane preparation (9), neuronal cells (18), retinal cells (44), human submandibular gland (HSG) cells (13), B lymphocytes (31), intestinal epithelial cells (34), mouse taste cells (8) and ovary cells (14). On the other hand, evidence from SH-SY5Y human neuroblastoma cells suggested that m-3M3FBS altered Ca\textsuperscript{2+} movement in a manner independent of PLC stimulation, and doubted the application of this chemical as a pharmacological tool to stimulate PLC (23). Therefore whether m-3M3FBS is a selective PLC activator is still controversial.

Ca\textsuperscript{2+} ions play a pivotal role in various biological events. A rise in intracellular free Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}) is a crucial trigger for numerous physiological and pathological responses in cells (4). However, an abnormal [Ca\textsuperscript{2+}]\textsubscript{i} rise often cause interference of ion flux, dysfunction of proteins, apoptosis, and proliferation, etc. (7). In this regard, m-3M3FBS was thought
to release store Ca^{2+} in rat primary cortical neuronal cultures and pheochromocytoma (PC12) cells (18); but the mechanism was unknown. In human renal Caki cancer cells, m-3M3FBS was suggested to evoke apoptosis via inducing a [Ca^{2+}]_{i} rise; however how this Ca^{2+} signal arose was unclear (22).

The effect of m-3M3FBS on [Ca^{2+}]_{i} in human prostate cancer cells has not been examined. We investigated the effect of this compound on [Ca^{2+}]_{i} in PC3 cells. The PC3 cell line is a useful model for prostate research. It has been shown that in this cell line, [Ca^{2+}]_{i} can increase in response to the stimulation of various ligands such as desipramine (6), safrole (5), capsazepine (15) and econazole (16).

In this study, fura-2 was used as a fluorescent Ca^{2+}-sensitive dye to measure [Ca^{2+}]_{i} changes. We show that m-3M3FBS induced concentration-dependent [Ca^{2+}]_{i} rises both in the presence and absence of extracellular Ca^{2+} in PC3 cells. The [Ca^{2+}]_{i} rises are characterized, the concentration-response plots in the presence and absence of extracellular Ca^{2+} are established, and the pathways underlying m-3M3FBS-evoked Ca^{2+} entry and Ca^{2+} release are explored.

**Materials and Methods**

**Cell Culture**

PC3 cells obtained from American Type Culture Collection were cultured in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

**Solutions Used in [Ca^{2+}]_{i} Measurements**

Ca^{2+}-containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl_{2}, 2 mM CaCl_{2}, 10 mM Hepes, and 5 mM glucose. M-3M3FBS was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other agents were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the solution used in experiments did not exceed 0.1%, and did not alter viability or basal [Ca^{2+}]_{i}.

**[Ca^{2+}]_{i} Measurements**

Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^{6}/ml. Cells were subsequently loaded with 2 µM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed with Ca^{2+}-containing medium twice and was made into a suspension in Ca^{2+}-containing medium at a density of 10^{7}/ml. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 ml cell suspension was added to 0.9 ml Ca^{2+}-containing or Ca^{2+}-free medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. During the recording, reagents were added to the cuvette by pausing the recording for 2 sec to open and close the cuvette-containing chamber. For calibration of [Ca^{2+}]_{i}, after completion of the experiments, the detergent Triton X-100 and 5 mM CaCl_{2} were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 µM) was subsequently added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. [Ca^{2+}]_{i} was calculated as previously described (11). Mn^{2+} quench of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 µM MnCl_{2}. MnCl_{2} was added to cell suspension in the cuvette 1 min before starting the fluorescence recording. Data were recorded at excitation signal at 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as described previously (29).

**Chemicals**

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). M-3M3FBS and other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

**Statistics**

Data are reported as typical or means ± SEM of three experiments. Data were analyzed by two-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®). Multiple comparisons between group means were performed by post-hoc analysis using the Tukey’s HSD (honestly significant difference) procedure. A P-value less than 0.05 was considered significant.

**Results**

Fig. 1A shows that the basal [Ca^{2+}]_{i} level was approximately 50 nM. At concentrations between 10 and 30 µM, m-3M3FBS evoked [Ca^{2+}]_{i} rises in a concentration-dependent manner in Ca^{2+}-containing medium. At 1 µM, m-3M3FBS did not cause a [Ca^{2+}]_{i} rise. The [Ca^{2+}]_{i} rise induced by 30 µM m-3M3FBS attained to 175 ± 2 nM (n = 3) followed by a slow decay.
The Ca\(^{2+}\) response saturated at 30 \(\mu\)M m-3M3FBS because at a concentration of 50 \(\mu\)M, m-3M3FBS induced a similar response as that induced by 30 \(\mu\)M. Fig. 1C (filled circles) shows the concentration-response plot of m-3M3FBS-induced \([\text{Ca}^{2+}]_i\) rises. The \([\text{Ca}^{2+}]_i\) rises evoked by 10-30 \(\mu\)M m-3M3FBS in Ca\(^{2+}\)-free medium are shown in Fig. 1B. At a concentration of 1 \(\mu\)M, m-3M3FBS did not cause a \([\text{Ca}^{2+}]_i\) rise. Removal of extracellular Ca\(^{2+}\) did not change the baseline, suggesting that the amount of leaked fura-2 from the cells was insignificant. At a concentration of 30 \(\mu\)M, m-3M3FBS evoked a \([\text{Ca}^{2+}]_i\) rise by 105 nM above baseline followed by a gradual decay. The concentration-response plot of m-3M3FBS-induced \([\text{Ca}^{2+}]_i\) rises in Ca\(^{2+}\)-free medium is shown in Fig. 1C (open circles). The EC\(_{50}\) value is approximately 15 \(\mu\)M.

Experiments were performed to confirm m-3M3FBS-induced \([\text{Ca}^{2+}]_i\) rise involved Ca\(^{2+}\) influx. Mn\(^{2+}\) enters cells through similar pathways as Ca\(^{2+}\).
but quenches fura-2 fluorescence at all excitation wavelengths (29). Thus, quench of fura-2 fluorescence excited at the Ca\textsuperscript{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm suggests Ca\textsuperscript{2+} entry. Fig. 2 shows that 30 µM m-3M3FBS evoked an immediate decrease in the 360 nm excitation signal (compared to trace a). This implies that m-3M3FBS-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise involved Ca\textsuperscript{2+} entry. The decrease attained to a maximum of 65 ± 2 units (n = 3) at the time point of 110 sec.

Experiments were further conducted to explore the Ca\textsuperscript{2+} entry pathway of the m-3M3FBS-induced response. Three store-operated Ca\textsuperscript{2+} influx inhibitors: nifedipine (1 µM), econazole (0.5 µM), SK&F96365 (5 µM); and aristolochic acid (20 µM; a phospholipase A2 inhibitor) partly inhibited 30 µM m-3M3FBS-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise. In contrast, phorbol 12-myristate 13-acetate (PMA; 10 nM; a protein kinase C activator) or GF109230X (2 µM; a protein kinase C inhibitor) had no effect on m-3M3FBS-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise (Fig. 3).

Previous studies have shown that the endoplasmic reticulum is the major Ca\textsuperscript{2+} store in PC3 cells (5, 6, 15, 16). Fig. 4A shows that in Ca\textsuperscript{2+}-free medium, after treatment with 30 µM m-3M3FBS, addition of 1 µM thapsigargin (TG), an inhibitor of endoplasmic reticulum Ca\textsuperscript{2+} pumps (39), evoked a [Ca\textsuperscript{2+}]\textsubscript{i} rise of 25 ± 2 nM (n = 3). Fig. 4B shows that addition of TG induced a [Ca\textsuperscript{2+}]\textsubscript{i} rise of 65 ± 3 nM (n = 3). Subsequently added m-3M3FBS (30 µM) induced a [Ca\textsuperscript{2+}]\textsubscript{i} rise of 21 ± 2 nM which was smaller than the control m-3M3FBS-induced response (120 ± 2 nM; Fig. 4A) by 82% (P < 0.05) in the maximal value. Similar experiments were repeated by using another inhibitor of endoplasmic reticulum Ca\textsuperscript{2+} pumps, 2,5-di-tert-butylhydroquinone (BHQ) (42). Fig. 4C shows that BHQ (50 µM) added after pretreatment with 30 µM m-3M3FBS failed to induce a [Ca\textsuperscript{2+}]\textsubscript{i} rise. In contrast, Fig. 4D shows that BHQ induced a [Ca\textsuperscript{2+}]\textsubscript{i} rise of 40 ± 2 nM (n = 3). Subsequently added 30 µM m-3M3FBS induced a [Ca\textsuperscript{2+}]\textsubscript{i} rise of 61 ± 2 nM which was smaller than the control m-3M3FBS-induced response (126 ± 3 nM; Fig. 4C) by 52% (P < 0.05) in the maximal value. Cyclopiazonic acid (CPA) was another inhibitor of endoplasmic reticulum Ca\textsuperscript{2+} pump (36). Fig. 4E shows that 50 µM CPA induced a [Ca\textsuperscript{2+}]\textsubscript{i} rise of 30 ± 2 nM (n = 3). Subsequently added m-3M3FBS induced a [Ca\textsuperscript{2+}]\textsubscript{i} rise of 22 ± 2 nM (n = 3) which was smaller than the control m-3M3FBS-induced response by 83% (P < 0.05).

PLC-dependent production of inositol 1,4,5-trisphosphate is a key process for releasing Ca\textsuperscript{2+} from the endoplasmic reticulum (4, 7). Because m-3M3FBS was able to release Ca\textsuperscript{2+} from the endoplasmic
Fig. 4. Intracellular Ca\(^{2+}\) stores of m-3M3FBS-induced Ca\(^{2+}\) release. Experiments were performed in Ca\(^{2+}\)-free medium. M-3M3FBS (30 µM), thapsigargin (TG, 1 µM), BHQ (50 µM) and cyclopiazonic acid (CPA, 50 µM) were added at time points indicated. Data are typical of three experiments.
reticulum, the role of PLC in this release was examined. U73122, a PLC inhibitor (19, 40), was used to see whether this enzyme was necessary for m-3M3FBS-induced Ca\(^{2+}\) release. Fig. 5A shows that ATP (10 µM) induced a [Ca\(^{2+}\)]\(_i\) rise of 101 ± 2 nM (n = 3). ATP is a PLC-dependent agonist of [Ca\(^{2+}\)]\(_i\) rise in most cell types (10, 41). It has been shown that PC3 cells express P2X and P2Y receptors (35). Fig. 5B shows that incubation with 2 µM U73122 did not change basal [Ca\(^{2+}\)]\(_i\) but abolished ATP-induced [Ca\(^{2+}\)]\(_i\) rises. This suggests that U73122 effectively suppressed PLC activity. Fig 5B also shows that addition of 30 µM m-3M3FBS after U73122 and ATP treatments caused a [Ca\(^{2+}\)]\(_i\) rise not different from control (1st column, m-3M3FBS-induced group).

Discussion

Ca\(^{2+}\) signaling plays a crucial role in the function of almost all cell types including prostate cancer cells. Li et al. (25) show that the amino-terminal peptide of Bax perturbs intracellular Ca\(^{2+}\) homeostasis to enhance apoptosis in prostate cancer cells. Liao et al. (26) suggest that extracellular Ca\(^{2+}\) acts as a candidate mediator of prostate cancer skeletal metastasis. M-3M3FBS has been shown to induce a significant activation of PLC at concentration > 25 µM and marked Ca\(^{2+}\) elevation in several cell lines and in vitro (1). In contrast to the spiky responses induced by 25 µM m-3M3FBS seen in the study of Bae et al. (1), and slowly developing Ca\(^{2+}\) elevations in SH-SY5Y cells (23), our data reveal that 20 µM m-3MFBS induced an immediate increase in [Ca\(^{2+}\)]\(_i\), followed by a slow decay. The mechanism of m-3M3FBS-induced [Ca\(^{2+}\)]\(_i\) rise was apparently different in PC3 cells and SH-SY5Y cells, in the latter the [Ca\(^{2+}\)]\(_i\) rise was not altered by removal of extracellular Ca\(^{2+}\); in contrast, our findings show that removal of Ca\(^{2+}\) reduced the m-3M3FBS-induced [Ca\(^{2+}\)]\(_i\) rise by more than 50%. In SH-SY5Y cells, it was also shown that 5 µM U73122 strongly inhibited m-3M3FBS-mediated Ca\(^{2+}\) release (by 78 ± 13%); however, our results suggest that 2 µM U73122 effectively inhibited ATP-induced [Ca\(^{2+}\)]\(_i\) rise without altering m-3M3FBS-induced [Ca\(^{2+}\)]\(_i\) rise.

Our study is the first to show that m-3M3FBS induced [Ca\(^{2+}\)]\(_i\) rise in PC3 cells and examined the underlying mechanisms. Our data show that m-3M3FBS induced a concentration-dependent [Ca\(^{2+}\)]\(_i\) rise in PC3 cells between 10 µM and 30 µM. Most of previous studies utilizing m-3M3FBS to activate PLC were at concentration > 25 µM. The data suggest that m-3M3FBS increased [Ca\(^{2+}\)]\(_i\) by depleting intracellular Ca\(^{2+}\) stores and causing Ca\(^{2+}\) influx from extracellular milieu because removing extracellular Ca\(^{2+}\) reduced more than 50% of m-3M3FBS-induced [Ca\(^{2+}\)]\(_i\) rises.

Removal of extracellular Ca\(^{2+}\) reduced the m-3M3FBS-induced response throughout the measurement period, suggesting that Ca\(^{2+}\) influx occurred during the whole stimulation period. The ability of m-3M3FBS to induce Ca\(^{2+}\) influx was also independently demonstrated by m-3M3FBS-induced Mn\(^{2+}\) quench of fura-2 fluorescence.
The mechanism of m-3M3FBS-induced Ca\(^{2+}\) influx was examined. The results suggest that m-3M3FBS might cause Ca\(^{2+}\) influx via stimulating store-operated Ca\(^{2+}\) entry which is induced by depletion of intracellular Ca\(^{2+}\) stores (32), based on the inhibition of m-3M3FBS-induced [Ca\(^{2+}\)], rise by nifedipine, econazole and SK&F96365. Nifedipine was originally thought to be a selective blocker of L-type voltage-gated Ca\(^{2+}\) channels. Recent evidence shows that nifedipine also blocks store-operated Ca\(^{2+}\) channels (12, 33, 43). Econazole has been shown to inhibit store-operated Ca\(^{2+}\) channels in different models (17). (12, 33, 43). Econazole has been shown to inhibit store-operated Ca\(^{2+}\) entry in (2), and enhances store-operated Ca\(^{2+}\) entry in (36). Furthermore, aris-tein VP1-induced Ca\(^{2+}\) entry was suppressed if PLA2 (28) suggest that human parvovirus B19 capsid pro-tein VPI-induced Ca\(^{2+}\) entry was suppressed if PL A2 activity was inhibited. Most importantly, recent evi-dence shows that PLA2 controls endothelial store-operated Ca\(^{2+}\) entry and vascular tone in intact aorta (2), and enhances store-operated Ca\(^{2+}\) entry in dystrophic skeletal muscle fibers (3). Singaravelu et al. (37) show that PLA2 mediates store-operated Ca\(^{2+}\) entry in rat cerebellar granule cells. Therefore, these studies are in keeping with our results that PLA2 activity was necessary for m-3M3FBS-evoked Ca\(^{2+}\) signal in PC3 cells. Unfortunately, there exists no specific inhibitors of store operated Ca\(^{2+}\) channels. Thus nifedipine, econazole and SKF96365 were not expected to fully block Ca\(^{2+}\) entry through these channels. The result that inhibition of PLA2 was more effective in inhibiting m-3M3FBS-induced Ca\(^{2+}\) entry might suggest that PLA2 played a crucial effect in the opening of store-operated Ca\(^{2+}\) channels.

Because activation of PLC produces IP\(_3\) and diacylglycerol, which stimulates PKC, the effect of regulation of PKC activity on m-3M3FBS-induced [Ca\(^{2+}\)], rise was examined. Neither activation nor inhibition of PKC changed m-3M3FBS-induced [Ca\(^{2+}\)], rise. One of the possible mechanisms that might cause m-3M3FBS-induced [Ca\(^{2+}\)], rise is that m-3M3FBS inhibited plasma membrane Ca\(^{2+}\) ATP pump so that cytosolic Ca\(^{2+}\) could not be pumped out of the cells and [Ca\(^{2+}\)], would rise via leaks in the plasma membrane.

Regarding the Ca\(^{2+}\) stores involved in m-3M3FBS-induced Ca\(^{2+}\) release, the TG/BHQ/CPA-sensitive endoplasmic reticulum stores might be the main stores because TG/BHQ/CPA pretreatment reduced a major part of m-3M3FBS-induced [Ca\(^{2+}\)], rise; and conversely, pretreatment with m-3M3FBS inhibited TG-induced [Ca\(^{2+}\)], rise and abolished BHQ-induced response. Other Ca\(^{2+}\) stores responsible for m-3M3FBS-induced Ca\(^{2+}\) release may include mitochondria (20). However the main drawback of previous reports that used the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone to deplete mitochondrial Ca\(^{2+}\) was that this treatment would alter cellular energy production and renders data interpretation difficult.

Furthermore, it seems that PLC-dependent pathways did not play a role in m-3M3FBS-induced Ca\(^{2+}\) release, since the response was not affected when PLC activity was inhibited by U73122. This is consistent with the evidence found in SH-SY5Y cells that 25 µM m-3M3FBS failed to activate PLC and did not stimulate inositol phosphate generation (23). How m-3M3FBS released Ca\(^{2+}\) from endoplasmic reticulum Ca\(^{2+}\) store was unclear. One possibility was that m-3M3FBS might act similarly to TG/BHQ/ CPA by inhibiting endoplasmic reticulum Ca\(^{2+}\) pumps.

Lee et al. (24) reported that m-3M3FBS induced apoptosis of monocytic leukemia cells via a [Ca\(^{2+}\)], rise. M-3M3FBS was thought to induce apoptosis through caspase activation in human renal Caki cancer cells. This apoptosis was attenuated by chelating intracellular Ca\(^{2+}\), suggesting that m-3M3FBS-in-duced [Ca\(^{2+}\)], rise lead to apoptosis (22).

Together, the data show that m-3M3FBS induced Ca\(^{2+}\) release from endoplasmic reticulum and also caused Ca\(^{2+}\) influx via store-operated Ca\(^{2+}\) entry in a PLC-independent, PLA2-dependent manner. Because a rise in [Ca\(^{2+}\)], can alter many cellular responses, caution should be applied in using 25 µM m-3M3FBS as a putative PLC activator.

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References


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