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SPECIAL REPORT

Phospholipase C activator *m*-3M3FBS affects Ca^{2+} homeostasis independently of phospholipase C activation

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In this study, we have investigated responses to the phospholipase C (PLC) activator *m*-3M3FBS in SH-SY5Y human neuroblastoma cells. As measured using fura-2, *m*-3M3FBS caused a slowly developing – full response was obtained within 4–6 min – Ca^{2+} elevation both in the presence and absence of extracellular Ca^{2+}, indicating Ca^{2+} release from intracellular stores, putatively from endoplasmic reticulum and mitochondria. PLC activity was also measured using two methods, the classical ion-exchange separation and the more novel fluorescent real-time method. In the time frame in which *m*-3M3FBS caused Ca^{2+} elevation (up to 7 min), no PLC activation was detected. Instead, more than 20 min were required to see any inositol phosphate generation in response to *m*-3M3FBS. *m*-3M3FBS also interfered with store-operated Ca^{2+} influx and Ca^{2+} extrusion. In conclusion, *m*-3M3FBS cannot be considered either potent or specific PLC activator.
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Abbreviations: CHO cells, Chinese hamster ovary cells; CPA, cyclopiazonic acid; DAG, diacylglycerol; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; GPCR, G-protein-coupled receptor(s); *m*- and *o*-3M3FBS, 2,4,6-trimethyl-N-(*m*/*o*-3-trifluoromethylphenyl) benzenesulfonamide; oxotremorine-M, oxo-M, *N,N,N*,-trimethyl-4-(2-oxo-1-pyrrolidinyl)-2-butyn-1-ammonium iodide; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; TBM, TES-buffered medium; TPA, 12-*o*-tetradecanoylphorbol 13-acetate; U-73122, 1-(6-[([17b]-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl)-1*H*-pyrrole-2,5-dione

Introduction The phospholipase C (PLC) family incorporates five subfamilies: PLCβ, PLCγ, PLCδ, PLCε and PLCζ (Saunders *et al*., 2002; reviewed in Rebecchi & Pentyala, 2000; Wing *et al*., 2003). The most thoroughly investigated subfamilies, PLCβ (PLCβ1–4) and PLCγs (PLCγ1–2), are central for the signalling of G-protein-coupled receptors (GPCR) and growth factor tyrosine kinase receptors, respectively. The more novel PLCε is regulated by small G-proteins such as Ras, Rap2B and Rho. The activation of the other novel types PLCδ (PLCδ1–4) and PLCζ is far less characterised. All the PLC isoforms require Ca^{2+} for their activity, although their Ca^{2+} sensitivity varies a lot.

PLC activation, especially that of PLCβs and PLCγs is central for the signalling of many receptors. However, molecular tools to directly pinpoint the role of PLC in cellular processes or to be used as lead compounds in drug design have been scarce. The compounds used today as PLC inhibitors have emerged in other contexts and have only later been identified as (rather nonspecific) inhibitors of the PLC family. For example neomycin, caffeine and U-73122 (1-[6-([(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1*H*-pyrrole-2,5-dione) have been have shown to display inhibition of PLC. The problems with each of these are that nonspecific effects occur in the concentration range needed for inhibition of PLC and that they are ineffective in some cells (see e.g. Taylor & Broad, 1998; Fredholm *et al*., 1999; Soriente *et al*., 1999; Okada *et al*., 2003). Especially bothersome is the Ca^{2+} release from endoplasmic reticulum (ER) induced by U-73122; thus, U-73122, when used in Ca^{2+} measurements, may give a false impression of inhibition of PLC when it instead has drained ER of Ca^{2+} (Taylor & Broad, 1998). Compounds, which would instead enhance PLC activity would also significantly increase our understanding of different PLC-activated pathways. Yet, no specific PLC activators were known until recently, when the compound *m*-3M3FBS (2,4,6-trimethyl-*N*-[*m*-3-trifluoromethylphenyl] benzenesulphonamide) was introduced (Bae *et al*., 2003). This compound was found through a screen of a chemical library for compounds that would stimulate superoxide generation in human neutrophils (Bae *et al*., 2003). It was found to induce similar elevations of cytosolic Ca^{2+} as GPCR stimulation and to stimulate inositol phosphate generation in intact cells (Bae *et al*., 2003). It also activated members of different PLC subfamilies in an *in vitro* assay. This tool has raised hopes to allow circumvention of receptor-mediated processes and to specifically dissect PLC-mediated signalling. We aimed to use it in this purpose to support our investigations of the relationship of Ca^{2+} release, store-operated Ca^{2+} influx and store refilling in SH-SY5Y human neuroblastoma cells. However, the results with *m*-3M3FBS were not as expected from a PLC activator, and we therefore set out to investigate the mechanism of its action.

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Methods *Cell culture* SH-SY5Y cells were cultured as described in Kukkonen *et al*. (1997) and Chinese hamster ovary (CHO) cells as described in Lund *et al*. (2000). For microfluorometry, the cells were grown on uncoated circular glass coverslips (diameter 25 mm; Knittel, Braunschweig, Germany) on plastic culture dishes (inner diameter 35 mm; Greiner). For radioactive inositol phosphate measurements, the cells were grown on circular plastic culture dishes (inner diameter, 85 mm; Greiner Bio-One GmbH, Frickenhausen, Germany). The confluence of the dishes − independent of the assay or whether the cells were transfected or not − was $\approx$70% by the time of the experiments.

To measure PLC activity towards PIP_2 (phosphatidylinositol-4,5-bisphosphate) in real-time, some SH-SY5Y cells were transfected to introduce fusion protein of enhanced green fluorescent protein (EGFP) and PH domain of PLCδ1. SH-SY5Y cells were grown on glass coverslips to 40–50% confluence. The dishes were washed with PBS and the cells were transfected using 24.5 μl Lipofectamine reagent (Invitrogen Co., Carlsbad, CA, U.S.A.) and 12.6 μg DNA (peGFP-C1-PLCdelta-PH; Stauffer *et al*., 1998) in 7 ml of OPTI-MEM (Gibco) for 5 h, after which the medium was replaced with normal culture medium. Measurements were performed 43 h later.

Chemicals Digitonin was from Merck AG (Darmstadt, Germany). Fura-2 acetoxymethyl ester was from Molecular Probes Inc. (Eugene, OR, U.S.A.) and oxotremorine-M (*N,N,N*,-trimethyl-4-[2-oxo-1-pyrrolidinyl]-2-butyn-1-ammonium iodide; oxo-M) and thapsigargin from RBI (Natick, MA, U.S.A.). *m*-3M3FBS was from Calbiochem (La Jolla, CA, U.S.A.) and Tocris Cookson (Bristol, U.K.) and cyclopiazonic acid (CPA), *o*-3M3FBS and U-73122 from Tocris. FCCP (carbonyl cyanide 4-[trifluoromethoxy] phenylhydrazone), oligomycin, rotenone and TPA (12-*o*-tetradecanoylphorbol 13-acetate) were from Sigma (St Louis, MO, U.S.A.) and *myo*-[2-^{3}H]inositol (1 mCi ml^{-1}) from Amersham Pharmacia Biotech (Buckinghamshire, U.K.).

Media TES-buffered medium (TBM) consisted of 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose and 20 mM 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino) ethane sulfonic acid (TES) adjusted to pH 7.4 with NaOH.

Microfluorometric measurements of Ca^{2+} Microfluorometric measurements of Ca^{2+} were performed using InCyt2™ fluorescence imaging system (Intracellular Imaging, Cincinnati, OH, U.S.A.). Briefly, the cells, cultured on glass cover slips, were loaded with 4 μM fura-2 acetoxymethyl ester for 20 min at 37°C. The cells were excited by alternating 340 and 380 nm light with the use of narrow band excitation filters and a filter wheel. The emission light was collected through a dichroic mirror and a 510 nm barrier filter with a CCD camera. The coverslips were constantly perfused at a rate of 1 ml min^{-1} in a $\approx$160 μl perfusion chamber at 37°C. The additions into the chamber were made by perfusion. The data were analysed using the InCyt2 software and Microsoft Excel.

PLC activity PLC activity was measured using two different methods. The first method was based on ion-exchange separation of total liberated inositol phosphates (Lund *et al*., 2000). The cells were prelabelled with 3 μCi ml^{-1} *myo*-[^{3}H]inositol for 20 h after which they were harvested. The cells were preincubated for 10 min in TBM containing 10 mM LiCl, whereafter they were stimulated for 20, 60 or 120 min. The reactions were terminated by spinning and replacement of the TBM with ice-cold perchloric acid. The total inositol phosphate fraction of the neutralised supernatants was isolated with anion-exchange chromatography.

The second method was based on the translocation of the fusion protein of EGFP and PH-domain of PLCδ1 from plasma membrane to cytosol upon PLC activation, which is a very specific measure of PIP_2 breakdown/IP_3 generation (Stauffer *et al*., 1998). Fluorescence was measured with a Nikon TE200 fluorescence microscope (100 ×/1.30 oil immersion objective), TILLvisION v. 4.01 imaging system (TILL Photonics GmbH, Gräfelding, Germany) and a high-resolution cooled CCD camera. The coverslips were constantly perfused at a rate of 2 ml min^{-1} in a $\approx$160 μl perfusion chamber at 37°C. The additions into the chamber were made by perfusion. The data were analysed using TILLvisION software and Microsoft Excel.

Results We first exposed SH-SY5Y loaded with fura-2 to 25 μM *m*-3M3FBS, a concentration that gives a significant activation of PLC (i.e. lower concentrations would not cause a useful PLC activation) and marked Ca^{2+} elevation in several cell lines and *in vitro* (Bae *et al*., 2003). In contrast to the strong, 'spiky', GPCR-like responses seen in the study of Bae *et al*. (2003), we observed slowly developing Ca^{2+} elevations upon exposure to 25 μM *m*-3M3FBS (Figure 1b; compare to a typical GPCR response *via* muscarinic receptors in Figure 1a). These Ca^{2+} elevations were similar both in the presence and in the absence of extracellular Ca^{2+} (Figure 1b). Sometimes spikes superimposed on the slow Ca^{2+} elevation were seen (grey trace, Figure 1b). Ca^{2+} release responses to muscarinic stimulation, thapsigargin and CPA were strongly but not completely inhibited by *m*-3M3FBS pretreatment (not shown). In contrast, the muscarinic response was completely attenuated after exposure to thapsigargin or CPA, which deplete ER Ca^{2+} stores (not shown), and *vice versa*. In cells pretreated with thapsigargin or CPA, however, some Ca^{2+} elevation in response to *m*-3M3FBS was still seen (Figure 1c). The remaining Ca^{2+} release is likely to originate from mitochondria as it was fully blocked by FCCP and strongly inhibited by rotenone + oligomycin pretreatment (Figure 1c). FCCP alone caused a Ca^{2+} release supporting the active role of mitochondria in Ca^{2+} signalling in SH-SY5Y cells. The subsequent response to *m*-3M3FBS was markedly but not fully inhibited, suggesting that *m*-3M3FBS induced Ca^{2+} release from both ER and mitochondria.

U-73122, at 5 μM, but not at lower concentrations, fully blocked the oxo-M-mediated Ca^{2+} release, but did not cause any obvious Ca^{2+} elevation itself, as 10 μM U-73122 did (not shown). However, Even 5 μM U-73122 interfered with ER, as indicated by the reduced response to thapsigarin/CPA. In addition, all the concentrations of U-73122 led to a complete loss of shape of cells (rounding up). In total, 5 μM U-73122 strongly inhibited *m*-3M3FBS-mediated Ca^{2+} release (by 78 $\pm$ 13%).

While measuring muscarinic responses there were indications of reduction of store-operated Ca^{2+} influx in

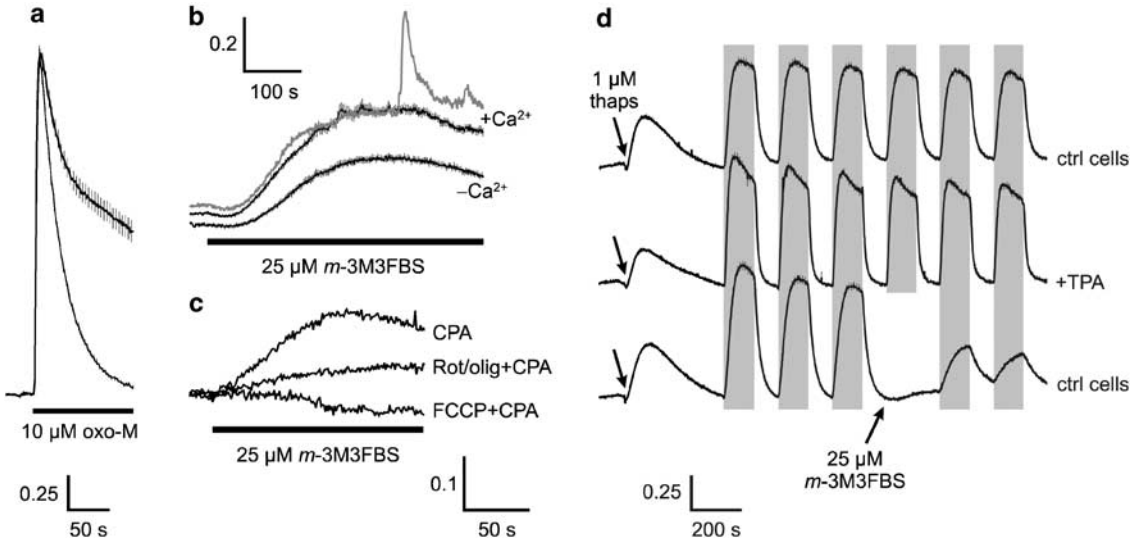

Figure 1 Ca^{2+} responses to muscarinic receptor stimulation (oxo-M) and *m*-3M3FBS. (a) Cells were stimulated with oxo-M in 1 mM extracellular Ca^{2+} ($+Ca^{2+}$) and in nominally Ca^{2+}-free TBM (no Ca^{2+} added; $-Ca^{2+}$). (b) Cells were stimulated with *m*-3M3FBS in similar conditions. The black traces represent average responses of 30 respectively 28 cells. The grey trace displays the response of one of the individual cells in 1 mM extracellular Ca^{2+}. The spike seen is spontaneous. (c) Cells were treated with CPA for 6 min before exposure to *m*-3M3FBS. CPA pretreatment was in some cases, as indicated, preceded by rotenone and oligomycin (6 min) or FCCP pretreatment (2 min). (d) Effect of *m*-3M3FBS on store-operated Ca^{2+} influx was assessed. The medium was nominally Ca^{2+}-free TBM except for the areas marked with grey boxes, which indicate 1 mM Ca^{2+}. In the beginning of each experiment, 1 μM thapsigargin was added, and was acting throughout the experiment. The cells in the middle trace were pretreated with 2 μM TPA for 10 min (also acting throughout the experiment). In the bottom trace, *m*-3M3FBS was added as indicated. The vertical calibration bars indicate change in ratio 340/380 nm (arbitrary units). The traces are averages $\pm$ s.e.m. of 18–53 cells; for the sake of clarity, error bars are only shown for every third (a, b) or 10th (d) point or not at all (c). Experiments were repeated with three coverslips each.

m-3M3FBS-treated cells (not shown). We therefore investigated this using thapsigargin treatment, which activates the store-operated influx in receptor- and PLC-independent manner. Even in this assay, the magnitude and the rate of the store-operated Ca^{2+} influx was much reduced by *m*-3M3FBS (Figure 1d; compare top and bottom traces). A simple analysis could not be applied on the rate of Ca^{2+} influx (neither first- nor second-order reaction), but the peak was obtained in control cells in 60–80 s, whereas this took more than 100 s in *m*-3M3FBS-treated cells. However, since the peak level of Ca^{2+} elevation was much reduced in *m*-3M3FBS-treated cells, the actual difference in the influx rate may be much greater. Also, the rate of Ca^{2+} efflux was decreased by *m*-3M3FBS (-3.38 ± 0.27 and $-0.88 \pm 0.04\,min^{-1}$ for ctrl and m-3M3FBS-treated cells, respectively; Figure 1d). These effects were unlikely to be caused by PLC activation and subsequent PKC activation, because the diacylglycerol analogue TPA instead enhanced both processes (Figure 1d; middle trace).

We subsequently investigated the ability of *m*-3M3FBS to activate PLC in SH-SY5Y cell. SH-SY5Y cells with healthy appearance and mainly plasma membrane GFP-PH-PLCδ1 fluorescence (Figure 2a) were chosen for the microfluorometric measurements. Upon PIP_2 hydrolysis, the GFP-PH-PLCδ1 fluorescence will translocate to the cytosol (Stauffer *et al*., 1998). Exposure of these cells to *m*-3M3FBS for up to 7 min did not cause any translocation of the fluorescence (Figure 2a,b and d). In contrast, muscarinic receptor stimulation with oxo-M ($G\alpha_q$ effect) caused a rapid and strong translocation of the fluorescence to the cytosol (Figure 2a,c and d).

We also investigated the PLC activation using the conventional radioactive method allowing longer incubations and devoid of the problems associated with SH-SY5Y cell motility. Also using this method, a clear response was observed with muscarinic receptor stimulation already after 20 min (Figure 2e). In contrast, no response to *m*-3M3FBS was seen before the later time points, 60 and 120 min. The responses to muscarinic receptor stimulation and *m*-3M3FBS were not additive. To investigate whether PLC activation by *m*-3M3FBS could be secondary to Ca^{2+} elevation, the cells were exposed to thapsigargin. However, this treatment did not stimulate inositol phosphate generation (Figure 2e). U-73122 effectively antagonised the PLC activation *via* muscarinic receptor, but surprisingly not by *m*-3M3FBS (Figure 2f).

We performed some additional experiments with the inactive analogue of *m*-3M3FBS, *o*-3M3FBS. *o*-3M3FBS was much weaker in releasing Ca^{2+} than *m*-3M3FBS, and it did not cause any inositol phosphate response.

In CHO cells, responses to *m*-3M3FBS very similar to those in SH-SY5Y cells were seen. *m*-3M3FBS caused a slowly developing Ca^{2+} elevation, although of lower magnitude than in SH-SY5Y cells. It also markedly but noncompletely reduced ER Ca^{2+} content. No inositol phosphate elevation was observed after 20 min stimulation.

Discussion In this study, we have investigated the effects of *m*-3M3FBS in SH-SY5Y human neuroblastoma cells, which have previously been utilised for numerous studies on GPCR, PLC and Ca^{2+} signalling. Ca^{2+} measurements showed a

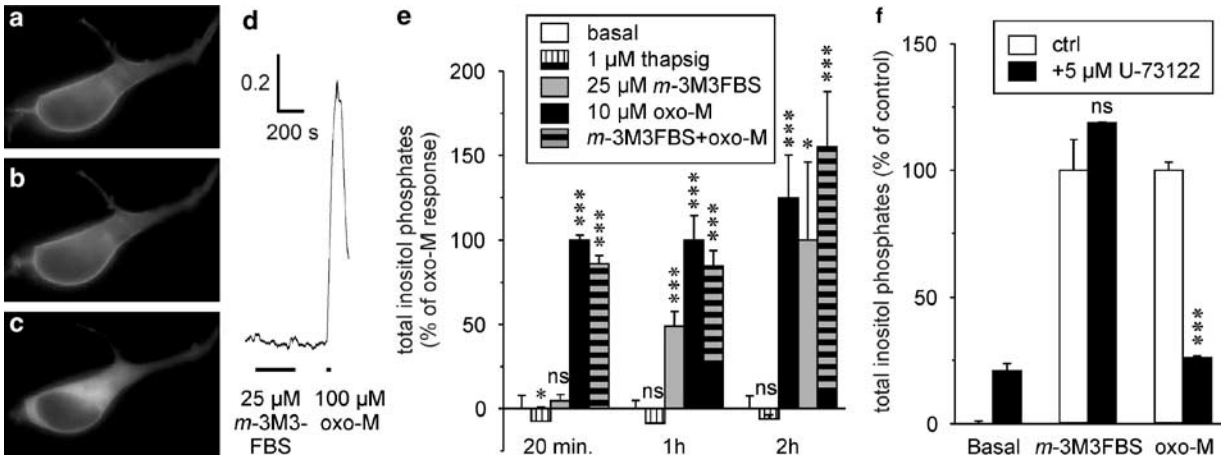

Figure 2 PLC activity as measured using two different techniques. (a-d) The GFP-PH-PLCδ1 probe binds to PIP_2 (membrane) and upon hydrolysis of this (i.e. generation of IP_3 and diacylglycerol), the probe translocates to the cytosol. (a) Cells before stimulation. (b) Cells were stimulated with 25 μM *m*-3M3FBS (picture 4 min after the addition of *m*-3M3FBS). (c) Cells were stimulated with 10 μM oxo-M (picture 10 s after the addition of oxo-M). (d) Time curve of the fluorescence changes in an individual cell. The vertical scale indicates cytosolic fluorescence divided by the membrane fluorescence (arbitrary units), that is, IP_3 production. The experiment was repeated with four coverslips. (e) Ion-exchange chromatographic separation of liberated ^{3}H-labelled inositol phosphates. The data were normalised to the response to 10 μM oxo-M at each time point. The data are from one experiment performed in triplicate and was performed twice with similar results. The significances are as follows: ns, not significant ($P>0.05$); *, $P<0.05$; ***, $P<0.001$.There was no significant difference between oxo-M and *m*-3M3FBS + oxo-M, except for 20 min (*). (f), the effect of the PLC inhibitor U-73122. The response to *m*-3M3FBS and oxo-M were measured after 2 h and 20 min, respectively. For the sake of clarity, the data are normalised to the basal level (0%) and to the maximum response to either *m*-3M3FBS or oxo-M in the absence of U-73122 (100%). Significances as in (e).

slowly developing (1–2 min lag, full magnitude within 4–6 min) Ca^{2+} release. Results suggest that this depends on both ER and mitochondria. Ca^{2+} release occurs in the time frame where no inositol phosphates are generated so *m*-3M3FBS should release Ca^{2+} *via* other mechanisms than IP_3. However, in an apparent contradiction, U-73122 markedly inhibited the Ca^{2+} response to *m*-3M3FBS. This is difficult to explain since no PLC activity is observed with the very sensitive methods used. A possible explanation could be that *m*-3M3FBS would cause a very minute IP_3 elevation that would be required as a trigger for the Ca^{2+} elevation then enhanced and prolonged by the other unspecific effects of U-73122 (see below). Another and more likely explanation is that U-73122 is toxic to the cells; we observed multiple toxic effects such as interference with Ca^{2+} release and storage in ER and mitochondria. Thus, the blocking of the *m*-3M3FBS response may not be due to the inhibition of PLC. Under any circumstances, it is clear that *m*-3M3FBS causes multiple effects that do not depend on PLC (e.g. decreased Ca^{2+} influx and efflux). It should also be noted that a long-lasting Ca^{2+} elevation in the absence of extracellular Ca^{2+} would not be expected from a PLC activator. Muscarinic receptors, which do not desensitise, and even the ER emptying substances thapsigargin and CPA, give a transient response in the absence of extracellular Ca^{2+}. In addition, PLC products should not interfere with mitochondrial function. Therefore, it is likely that the interference with Ca^{2+} extrusion and storage/buffering play an important part in the response to *m*-3M3FBS.

One possible explanation to the Ca^{2+} responses comes from the original study (Bae *et al*., 2003). *m*-3M3FBS was isolated as a compound able to induce superoxide production, but the relationship between this and PLC activation or the sensitivity of superoxide production to U-73122 was not investigated. Ability of *m*-3M3FBS to induce production of oxygen-free radicals independently of PLC activation could explain most of its effects on Ca^{2+} signalling (see for instance Suzuki *et al*., 1997). Sensitisation of the IP_3 receptor with oxygen free radicals for spontaneous IP_3 spikes might even offer an explanation to the apparently contradictory finding of the inhibitory effect of U-73122 on Ca^{2+} release by *m*-3M3FBS. However, this is highly speculative.

We cannot explain why *m*-3M3FBS effectively activates PLC in some and not in other cells. Obviously, the low efficacy of *m*-3M3FBS as a PLC activator in SH-SY5Y (and CHO) cells is not caused by low membrane penetration, since marked Ca^{2+} responses are observed within few minutes of exposure. It also is unlikely to be selective for particular isoforms of PLC.

In conclusion, the results demonstrate that *m*-3M3FBS is not acting as a potent or selective PLC activator in SH-SY5Y cells, but affects Ca^{2+} signalling primarily *via* other mechanisms. No PLC activation is measurable in the same time frame as Ca^{2+} responses, and even a putative PLC activation would not be able to explain the different effects on Ca^{2+} metabolism.

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