Single-cell imaging of graded Ins(1,4,5)P3 production following G-protein-coupled-receptor activation

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The pleckstrin homology domain of phospholipase Cβ1 (PHβ1,CHO) binds Ins(1,4,5)P3 and PtdIns(4,5)P2 specifically, and can be used to detect changes in Ins(1,4,5)P3 in single cells. A fusion construct of PHβ1,CHO and enhanced green fluorescent protein (EGFP–PHβ1,CHO) associates with the plasma membrane due to its association with PtdIns(4,5)P2. However, PHβ1,CHO has greater affinity for Ins(1,4,5)P3 than PtdIns(4,5)P2, and translocates to the cytosol as Ins(1,4,5)P3 levels rise. Prolonged activation of group I metabotropic glutamate receptor 1z expressed in Chinese-hamster ovary cells or endogenous M3 muscarinic receptors in SH-SY5Y neuroblastoma cells gave an initial transient peak in translocation, followed by a sustained plateau phase. This closely followed changes in cell population Ins(1,4,5)P3 mass, but not PtdIns(4,5)P2 levels, which decreased monophasically, as determined by radioreceptor assay. Translocation thus provides a real-time method to follow increases in Ins(1,4,5)P3. Graded changes in Ins(1,4,5)P3 in Chinese-hamster ovary-lac-mGlu1z cells could be detected with increasing glutamate concentrations, and dual loading with fura 2 and EGFP–PHβ1,CHO showed that changes in intracellular Ca2+ concentration closely paralleled Ins(1,4,5)P3 production. Moreover, Ins(1,4,5)P3 accumulation and intracellular Ca2+ mobilization within single cells is graded in nature and dependent on both agonist concentration and receptor density.

Key words: group I metabotropic glutamate receptor 1z, M3 muscarinic receptor, pleckstrin homology domain.

INTRODUCTION

The ability to determine intracellular calcium concentrations ([Ca2+]i) in single cells has had a profound effect on our understanding of the dynamics of G-protein-coupled-receptor (GPCR)-stimulated mobilization of intracellular calcium (Ca2+) stores. Imaging techniques have made it possible to visualize oscillations in [Ca2+]i, which paralleled Ins(1,4,5)P3 levels rise. Prolonged activation of group I metabotropic glutamate receptor 1z expressed in Chinese-hamster ovary cells or endogenous M3 muscarinic receptors in SH-SY5Y neuroblastoma cells gave an initial transient peak in translocation, followed by a sustained plateau phase. This closely followed changes in cell population Ins(1,4,5)P3 mass, but not PtdIns(4,5)P2 levels, which decreased monophasically, as determined by radioreceptor assay. Translocation thus provides a real-time method to follow increases in Ins(1,4,5)P3. Graded changes in Ins(1,4,5)P3 in Chinese-hamster ovary-lac-mGlu1z cells could be detected with increasing glutamate concentrations, and dual loading with fura 2 and EGFP–PHβ1,CHO showed that changes in intracellular Ca2+ concentration closely paralleled Ins(1,4,5)P3 production. Moreover, Ins(1,4,5)P3 accumulation and intracellular Ca2+ mobilization within single cells is graded in nature and dependent on both agonist concentration and receptor density.

Recent developments to track the subcellular localization of green fluorescent protein (GFP)-tagged proteins with high selectivity for intracellular messengers now make it possible to image cellular levels of Ins(1,4,5)P3 in real time in single cells [14–16]. The pleckstrin homology domain of PLCβ1 (PHβ1,CHO) binds with high affinity and selectivity to PtdIns(4,5)P2 [17], and a fusion construct of PHβ1,CHO with enhanced GFP (EGFP–PHβ1,CHO) enriches over the plasma membrane through this association [14–16]. Recently [16], PHβ1,CHO was found to show approx. 20-fold greater affinity for the soluble head-group of PtdIns(4,5)P2, i.e. Ins(1,4,5)P3, using a surface plasmon assay. Moreover, intracellular injection of Ins(1,4,5)P3 elicited translocation of the fusion protein to the cytosol, and co-transfection with the catabolic enzyme Ins(1,4,5)P3 5-phosphatase prevented agonist-induced translocation [16]. The combined data suggest, therefore, that agonist-stimulated Ins(1,4,5)P3 production is primarily responsible for translocation of EGFP–PHβ1,CHO with little contribution of PtdIns(4,5)P2 depletion as a result of PLC activity.

Using our knowledge of M3 muscarinic receptor-induced changes in the levels of Ins(1,4,5)P3 and its phosphoinositide precursor in SH-SY5Y neuroblastoma cells [18–20], we provide further evidence that the extent of membrane association of EGFP–PHβ1,CHO reflects cellular Ins(1,4,5)P3 levels. We have used this technique to study single-cell Ins(1,4,5)P3 production following activation of a group I metabotropic glutamate receptor 1z (mGlu1α) heterologously expressed in Chinese-hamster ovary (CHO) cells [21,22]. In the present paper, we demonstrate for the first time that Ins(1,4,5)P3 production is graded in response to changes in agonist concentration within individual cells and that...
levels closely correlate with changes in $[Ca^{2+}]_i$. We conclude that EGFP–PH$_{PLC\delta}$ translocation represents an excellent index to follow Ins(1,4,5)P$_3$ production and is likely to have a major impact on our understanding of GPCR-stimulated PLC activity.

**EXPERIMENTAL**

Vector containing the fusion construct between EGFP and the PH domain of PLC$_\delta$1 was kindly provided by Professor T. Meyer (Stanford University, CA, U.S.A.). Detailed information regarding this construct can be found in Stauffer et al. [14]. A description of the LacSwitch II-inducible expression system (Stratagene) used to express human mGlu1$_a$ receptor in CHO cells (CHO-lac-mGlu1$_a$) [21], and a comprehensive pharmacological analysis of the endogenous M$_2$ muscarinic receptor expressed by SH-SY5Y cells [23,24], are provided elsewhere. Information regarding the culture conditions for the two cell types is given in the relevant references.

Single-cell measurement of Ins(1,4,5)P$_3$ was conducted on cells seeded on to 22 mm diameter borosilicate coverslips. Cells were incubated for 8 h and then transiently transfected with EGFP–PH$_{PLC\delta}$ plasmid DNA using FuGENE 6$^{TM}$ (1:3, w/v), as per manufacturer’s instructions (Roche Diagnostics, Lewes, East Sussex, U.K.). For CHO-lac-mGlu1$_a$ cells, after 20 h the transfection medium was replaced with medium containing 100 $\mu$M isopropyl-$\beta$-d-thiogalactoside (IPTG) and the cells were incubated for another 20 h. Cells were perfused (5 ml/min) with Krebs-Henseleit buffer (KHB; 10 mM Hepes/118 mM NaCl/4.69 mM KCl/10 mM glucose/1.18 mM KH$_2$PO$_4$/4.2 mM NaHCO$_3$/1.18 mM MgCl$_2$/1.3 mM CaCl$_2$, pH 7.4) using a Gilson Minipuls 2 pump connected to a coverslip chamber maintained at 37°C using a Peltier unit, and confocal images were captured using an UltraVIEW LCI confocal system (Perkin Elmer). Drug solutions were either applied through the perfusion line or, for shorter (<30 s) periods of challenge, directly perfused over the cells with the outflow and inflow perfusion lines open and closed respectively.

For dual imaging of Ins(1,4,5)P$_3$ and $[Ca^{2+}]_i$, transfected cells were also loaded with 5 $\mu$M fura 2 acetoxyethyl ester and mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. Sequential images were captured at wavelengths above 510 nm after excitation at 340, 380 and 490 nm using an intensified charge-coupled device camera (Photonic Science) connected to a Quanticell 700 (Applied Imaging, Sunderland, Tyne and Wear, U.K.) system. To analyse the EGFP–PH$_{PLC\delta}$ signal, an area within the cytoplasm was highlighted and the mean fluorescence recorded. The data are expressed as a ratio of fluorescence at a given time point to basal levels. $[Ca^{2+}]_i$, from the same region was determined as described previously [25].

Radioreceptor assays for quantification of Ins(1,4,5)P$_3$ mass and determination of PtdIns(4,5)P$_2$ levels in cell populations were performed as described by Willars et al. [20]. Statistical analysis was performed using Student’s $t$ test, and $P$ values < 0.05 were considered statistically significant.

**RESULTS**

Transient transfection of CHO-lac-mGlu1$_a$ (Figure 1A) and SH-SY5Y (Figure 1C) cells with EGFP–PH$_{PLC\delta}$ resulted in a concentration of fluorescence over the plasma membrane representing the association of the fusion protein with PtdIns(4,5)P$_2$.

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Figure 1  Confocal single-cell imaging of agonist-induced changes in EGFP–PH$_{PLC\delta}$ fluorescence in CHO-lac-mGlu1$_a$ and SH-SY5Y cells

CHO-lac-mGlu1$_a$ (A, B) and SH-SY5Y neuroblastoma (C, D) cells transiently transfected with EGFP–PH$_{PLC\delta}$ were perfused with either 1 mM glutamate or 1 mM carbamol respectively, and fluorescent images captured prior to (A, C) and approx 60 s after (B, D) drug challenge. An animation showing the change in cytosolic EGFP–PH$_{PLC\delta}$ fluorescence in CHO-lac-mGlu1$_a$ cells during glutamate application can be viewed at http://www.BiochemJ.org/bj/356/bj3560137add.htm.
Challenge with maximal concentrations of glutamate (Figure 1B) or carbachol (Figure 1D) resulted in a loss of membrane association and an enrichment of cytosolic fluorescence in CHO-lac-mGlu1x and SH-SY5Y cells, respectively. An animation showing the change in cytosolic EGFP–PHPLC fluorescence in CHO-lac-mGlu1x cells during glutamate application can be viewed at http://www.BiochemJ.org/bj/356/bj3560137add.htm. Expression of the EGFP control plasmid in SH-SY5Y cells resulted in cytosolic fluorescence only, and this was unchanged following methacholine treatment (results not shown).

Plotting the change in cytosolic fluorescence against time during prolonged agonist challenge revealed a large initial peak followed by a lower sustained level in the CHO-lac-mGlu1x and SH-SY5Y cells (Figure 2). These changes in fluorescence ratio returned to basal levels after perfusion with KHB alone. This pattern is typical for the stimulation of Ins(1,4,5)P$_3$ production (Figure 3A), quantified using a radioreceptor assay, following activation of M$_3$ muscarinic receptors in populations of SH-SY5Y cells where agonist was either washed out or 2 μM atropine added. In contrast, levels of PtdIns(4,5)P$_2$ were found to decrease monophasically in the SH-SY5Y cells during agonist challenge, with no evidence of an inverted ‘peak’ and ‘plateau’ response (Figure 3B). Graded EGFP–PHPLC translocation in CHO-lac-mGlu1x cells was obtained to increasing concentrations of glutamate applied for 30 s (Figures 4A and 4B), and the graded nature of the response was confirmed by the combined data from different cells (Figure 4C). Similar results were obtained in a separate series of experiments where glutamate was perfused over the cells for 3 min and peak responses determined (results not shown). An animated version of the experiment shown in Figure 4A can be viewed at http://www.BiochemJ.org/bj/356/bj3560137add.htm.

Dual measurement of Ins(1,4,5)P$_3$ concentration and [Ca$^{2+}$] in glutamate-challenged CHO-lac-mGlu1x cells revealed the close relationship between these two consequences of PLC activation (Figure 5). The ratiometric values for EGFP–PHPLC changes were less than those obtained previously (Figure 2) because interference from different focal planes increases the background fluorescence in these non-confocal experiments. Prolonged exposure to glutamate (1 mM) induced a peak and plateau in both Ins(1,4,5)P$_3$ concentration and [Ca$^{2+}$], (Figure 5A). The changes in [Ca$^{2+}$] were consistently observed to peak and reach sustained levels before those in Ins(1,4,5)P$_3$ concentration (Figure 5A), although it is possible that this may only reflect differences in the kinetics of the two detection methods. Concentration-dependency for both the initial peak height and plateau level (measured at 200 s) of glutamate-induced changes in Ins(1,4,5)P$_3$ and [Ca$^{2+}$] were observed (Figure 5B). EC$_{50}$ values of 7.4 μM and 3.3 μM for the peak Ins(1,4,5)P$_3$ and [Ca$^{2+}$] response were obtained respectively, and similar values for the plateau levels of
peak Ins(1,4,5)P$_3$ concentration and [Ca$^{2+}$], responses by IPTG were 20 nM and 7 nM respectively. Plateau $E_{	ext{Ca}}$ values were 22 nM and 5 nM for Ins(1,4,5)P$_3$ concentration and [Ca$^{2+}$], respectively. Thus the extent of Ins(1,4,5)P$_3$ production and Ca$^{2+}$ mobilization is determined not only by agonist concentration, but also by the level of expression of the receptor. Moreover, the relationship between the two parameters remains similar as receptor density decreases.

**DISCUSSION**

The crucial finding of the present work is that increasing activation of either M$_1$ muscarinic or mGlu1 receptors leads to finely graded increases in Ins(1,4,5)P$_3$ levels within a single cell. Previously this could only be inferred from population-based biochemical assays, and the development of a single-cell based Ins(1,4,5)P$_3$ assay has allowed this issue to be directly addressed for the first time. There is already strong evidence that assessing translocation of the EGFP–PH$_{	ext{PLC}}$ fusion protein is monitoring changes in Ins(1,4,5)P$_3$ rather than measuring the loss of membrane PtdIns(4,5)P$_2$ due to hydrolysis by PLC [16]. Thus PH$_{	ext{PLC}}$ shows 20-fold higher affinity for Ins(1,4,5)P$_3$ than PtdIns(4,5)P$_2$, and intracellular injection of Ins(1,4,5)P$_3$ induces EGFP–PH$_{	ext{PLC}}$ translocation even in cells where Ca$^{2+}$ stores were depleted to eliminate the possibility of Ca$^{2+}$-induced PLC activation [16]. Furthermore, rapid catalysis of Ins(1,4,5)P$_3$ by transfection with an Ins(1,4,5)P$_3$ 5-phosphatase abolished agonist-induced EGFP–PH$_{	ext{PLC}}$ translocation [16].

We have further addressed this issue by comparing EGFP–PH$_{	ext{PLC}}$ translocation in SH-SY5Y cells with experiments measuring changes in Ins(1,4,5)P$_3$ and PtdIns(4,5)P$_2$ mass using radioreceptor-based assays of which our laboratory has extensive experience [20]. With this knowledge, we postulated that the changes in subcellular localization of EGFP–PH$_{	ext{PLC}}$ would mimic most closely the changes in Ins(1,4,5)P$_3$ or PtdIns(4,5)P$_2$ that mediate translocation. The data demonstrated that changes in cytosolic EGFP–PH$_{	ext{PLC}}$ fluorescence most closely paralleled the biphasic response characteristic of Ins(1,4,5)P$_3$ levels rather than the monophasic changes in PtdIns(4,5)P$_2$ levels (Figures 2B and 3).

Overall, this provides compelling evidence that EGFP–PH$_{	ext{PLC}}$ allows us to follow the levels of Ins(1,4,5)P$_3$ in a single cell in real time. The observation that EGFP–PH$_{	ext{PLC}}$ partially returns to the membrane during the plateau phase, at a time when PtdIns(4,5)P$_2$ levels remain low, has an additional implication. It may indicate that despite the marked overall decrease in PtdIns(4,5)P$_2$ concentration, ‘local’ levels of this phosphoinositide are still sufficient for a predominant plasma membrane localization of EGFP–PH$_{	ext{PLC}}$. Therefore the level of EGFP–PH$_{	ext{PLC}}$ expression must be small compared with total PtdIns(4,5)P$_2$ concentration. This further suggests that significant sequestration of Ins(1,4,5)P$_3$ by binding to PH$_{	ext{PLC}}$ is unlikely, and argues that, in an analogous manner to the use of fluorescent Ca$^{2+}$-sensing dyes, levels of EGFP–PH$_{	ext{PLC}}$ used to detect signalling events do not significantly influence the magnitude of the changes.

The demonstration of graded Ins(1,4,5)P$_3$ production has important implications for the understanding of the phenomenon of quantal Ca$^{2+}$ release. Thus in each cell a given agonist concentration elicits a defined and apparently finely tuned (Figure 4) amount of Ins(1,4,5)P$_3$ production. This sensitizes, in an Ins(1,4,5)P$_3$ concentration-dependent manner, a set number of Ins(1,4,5)P$_3$ receptors on the endoplasmic reticulum, resulting in specific quantal Ca$^{2+}$ release [26,27]. The magnitude of Ca$^{2+}$ release will be determined by the extent of interaction of Ins(1,4,5)P$_3$ with its receptor, combined with the ability of Ca$^{2+}$

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**Figure 4** Effect of incremental glutamate concentrations on Ins(1,4,5)P$_3$ levels in single CHO-lac-mGlu1 cells

CHO-lac-mGlu1 cells were transiently transfected with EGFP–PH$_{	ext{PLC}}$ for 48 h and receptor expression induced with 100 μM IPTG for 20 h. A series of confocal images of a single cell were captured following repeated challenge for 30 s with increasing concentrations of glutamate (A). An animated version of (A) can be viewed at http://www.BiochemJ.org/bj/356/bj3560137add.htm. Cells were washed with KHB for 150 s between each treatment. The changes in cytosolic EGFP–PH$_{	ext{PLC}}$ fluorescence in this cell are plotted in (B) and the data averaged (means±S.E.M.) for 10 separate cells from four independent experiments (C). Glutamate (100 μM) was determined to induce maximal responses in a separate series of experiments (results not shown).

Ins(1,4,5)P$_3$ (10.8 μM) and [Ca$^{2+}$], (2.6 μM). Graded responses could also be detected when making dual measurements (Figure 5C). Application of 10 μM glutamate to CHO-lac-mGlu1 cells induced a peak Ins(1,4,5)P$_3$ accumulation of 1.13±0.02 (n = 14; change in cytosolic EGFP–PH$_{	ext{PLC}}$ fluorescence, arbitrary units) and a peak of 415±56 nM above basal in [Ca$^{2+}$]. The same cells challenged with 1 mM glutamate responded with a peak of 1.23±0.03 (P < 0.001 compared with 10 μM) in Ins(1,4,5)P$_3$ accumulation and a 551±35 nM (P < 0.01) increase above basal in [Ca$^{2+}$]. Repeated application of glutamate (1 mM) for 3 s interspersed with 30 s washes demonstrated how closely the changes in [Ca$^{2+}$] and Ins(1,4,5)P$_3$ concentration paralleled each other (Figure 5D).

Using the inducible LacSwitch II-expression system, the levels of mGlu1 receptor present in the CHO-lac-mGlu1 cells can be controlled by simply varying the IPTG concentration [21,22]. Challenging cells exposed to IPTG for 20 h with 1 mM glutamate gave changes in both peak and plateau Ins(1,4,5)P$_3$ concentration (Figure 6A) and [Ca$^{2+}$] (Figure 6B), the magnitude of which varied according to the concentration of IPTG used to induce receptor expression. Half-maximal values for the induction of
once released to promote further Ca\(^{2+}\) mobilization through Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Interestingly, increases in Ins(1,4,5)\(P_3\) production are detectable over a relatively small range of agonist concentrations, such that relatively large variations can be observed between differences of a few \(\mu\)M. This is likely to be a consequence of feed-forward from the Ca\(^{2+}\) released from the intracellular stores on Ca\(^{2+}\)-activated PLCs (e.g. PLC\(\delta\) [11–13]) and facilitation of agonist-activated PLCs [11]. Indeed, we have previously shown [28] that Ca\(^{2+}\) store-depletion by thapsigargin pretreatment causes attenuations of agonist-mediated Ins(1,4,5)\(P_3\) responses to activation of M\(_1\) muscarinic receptors.

Dual imaging of [Ca\(^{2+}\)], and Ins(1,4,5)\(P_3\) showed that there is a close relationship between these two parameters. Not only do changes in levels parallel each other during prolonged agonist stimulation, but also repeated challenges elicit concurrent effects on both cytosolic Ins(1,4,5)\(P_3\) and Ca\(^{2+}\) levels. Moreover, graded changes in both Ins(1,4,5)\(P_3\) concentration and [Ca\(^{2+}\)], can be observed in CHO-lac-mGlu1z cells when the gluamate concentration is decreased. For the mGlu1z receptor we also show that there is little amplification of the response to glutamate between Ins(1,4,5)\(P_3\) production and [Ca\(^{2+}\)], release, since EC\(_{50}\) values differ by only 0.5 log unit. This contrasts markedly with M\(_1\) muscarinic-induced changes in Ins(1,4,5)\(P_3\) concentration and [Ca\(^{2+}\)], in SH-SY5Y cells, where EC\(_{50}\) values differ by approx. 2.0 log units [18]. This is likely to reflect differences in the extent of amplification between Ins(1,4,5)\(P_3\) production and [Ca\(^{2+}\)], release in a cell- and receptor-dependent manner. The importance of receptor density on mGlu1z receptor responses was also highlighted by titrating expression in cells challenged with a high concentration of glutamate. These results compare favourably with the extent of IPTG-induced mGlu1z receptor protein expression observed by Western blotting and with agonist-induced changes in Ins(1,4,5)\(P_3\) production determined using a radioreceptor assay [21, 22].

The use of EGFP–PH\(_{PLC}\) translocation to monitor cellular Ins(1,4,5)\(P_3\) levels has several clear advantages over traditional biochemical measurements made on cell populations. First, and most importantly, it provides data on single cells, and so gives the actual changes in each individual cell, rather than a summation of the effect on a population. This is crucial in order to distinguish between graded responses and changes in synchronicity, or in the number of responding cells. A corollary of this first point is that weak stimuli, which are undetectable biochemically because of asynchronicity, e.g. when responses oscillate, or when studying heterogeneous cell populations (i.e. mixed neuronal cultures), become detectable using EGFP–PH\(_{PLC}\) translocation. A second advantage is the ability to apply drugs by perfusion and measure continuously. This allows for repeated application/removal of drugs over widely variant time frames, while constantly acquiring data. Clearly, this is an extremely attractive approach to study regulation of GPCR-induced responses and to acquire temporal data on Ins(1,4,5)\(P_3\) production.

**Figure 5 Co-detection of changes in Ins(1,4,5)\(P_3\) and [Ca\(^{2+}\)], levels in single CHO-lac-mGlu1z cells stimulated with glutamate**

(A) Representative traces showing the simultaneous detection of changes in Ins(1,4,5)\(P_3\) concentration (solid line) and [Ca\(^{2+}\)], (dotted line) in a single CHO-lac-mGlu1z cell challenged with 1 mM glutamate (broken line). (B) Concentration-response curves for the effect of glutamate on peak (filled symbols) and plateau (open symbols) levels of Ins(1,4,5)\(P_3\) (circles) and [Ca\(^{2+}\)], (squares). A comparison of the changes in Ins(1,4,5)\(P_3\) (solid line) and [Ca\(^{2+}\)], (dashed line) levels induced by 1 mM glutamate with that for 10 \(\mu\)M glutamate is shown in (C). (D) The close relationship between Ins(1,4,5)\(P_3\) (top line) and [Ca\(^{2+}\)], (bottom line) following repeated challenge with 1 mM glutamate for 3 s interspersed with washing with KHB for 27 s.

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Figure 6 Effect of mGlu1α receptor density on stimulated Ins(1,4,5)P$_3$ and [Ca$^{2+}$], levels in single CHO-lac-mGlu1α cells

CHO-lac-mGlu1α cells were transiently transfected with EGFP–PH PLC$_{a}$ for 48 h and receptor expression induced by incubation with different concentrations of IPTG for 20 h. Cells were then loaded with fura 2 and challenged with 1 mM glutamate and the effect of different mGlu1α receptor density on peak (A) and plateau (B) levels of Ins(1,4,5)P$_3$ (A) and [Ca$^{2+}$]$_i$ (B) in single cells determined. Data are means ± S.E.M. and were compiled from > 6 separate cells from 2–4 experiments.

In conclusion, GPCR-induced translocation of EGFP–PH PLC$_{a}$ in single cells offers an excellent method to monitor Ins(1,4,5)P$_3$ accumulation in single cells. Thus Ins(1,4,5)P$_3$ levels in individual cells do follow a peak and plateau pattern and responses are dependent on both agonist concentration and receptor density.

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