Cellular delivery and photochemical release of a caged inositol-pyrophosphate induces PH-domain translocation in cellulo

Inositol pyrophosphates, such as diphospho-myo-inositol pentakisphosphates (InsP7), are an important family of signalling molecules, implicated in many cellular processes and therapeutic indications including insulin secretion, glucose homeostasis and weight gain. To understand their cellular functions, chemical tools such as photocaged analogues for their real-time modulation in cells are required. Here we describe a concise, modular synthesis of InsP7 and caged InsP7. The caged molecule is stable and releases InsP7 only on irradiation. While photocaged InsP7 does not enter cells, its cellular uptake is achieved using nanoparticles formed by association with a guanidinium-rich molecular transporter. This novel synthesis and unprecedented polyphosphate delivery strategy enable the first studies required to understand InsP7 signalling in cells with controlled spatiotemporal resolution. It is shown herein that cytoplasmic photouncaging of InsP7 leads to translocation of the PH-domain of Akt, an important signalling-node kinase involved in glucose homeostasis, from the membrane into the cytoplasm.
Diphospho-inositol polyphosphates (InsP$_2$) are second messengers involved in essential cell signalling pathways. A distinct difference of InsP$_7$ compared with other inositol polyphosphates is the presence of a phosphoanhydride bond in, for example, the 5-position (5-InsP$_7$, Fig. 1), rendering them a structurally unique class of second messengers. This special feature is also the reason for their nickname ‘inositol pyrophosphates’. InsP$_2$ are implicated in the regulation of diverse cellular and metabolic functions in different kingdoms of life. It has been proposed that InsP$_2$ bind to the pleckstrin homology (PH) domain of protein kinase B (Akt), and competitively suppress its specific phosphatidylinositol 3,4,5-trisphosphate (PtdIns-P$_3$) association at the plasma membrane, thereby inhibiting phosphoinositide-dependent kinase 1 (PDK1)-mediated phosphorylation of Akt. However, there remains uncertainty as to whether the reduced phosphorylation of Akt is a result of the inhibition of its membrane association via its PH-domain, since the in vitro assays that have been performed do not contain any membrane or membrane mimics. In addition, InsP$_2$ might act either as allosteric inhibitors or as non-enzymatic phosphorylating agents or both. Notwithstanding, inhibition of the Akt pathway by InsP$_2$ has an impact on glucose uptake and insulin sensitivity, as exemplified by a mouse model that lacks inositol hexakisphosphate-kinase 1 (IP6K1). These knockout mice have reduced levels of InsP$_7$ and show a lean phenotype on a high-fat diet concomitant with increased insulin sensitivity. To address this problem, a novel strategy based on the development of a levulinate benzyl ester adaptor (LevB) is described. The introduction of this new protecting group and its combination with fluorenylmethyl (Fm) protection and photocage introduction enables the previously inaccessible synthetic route to the first photoactivatable diphospho-inositol InsP$_7$ probe equipped with a [7-(diethylamino)-coumarin-4-yl]methyl (DEACM) photocage (Fig. 2a). It is noteworthy that this strategy potentially facilitates the introduction of other tags, such as, for example, photoaffinity labels and fluorophores.

The synthesis commenced with benzylidene protected 2 prepared as previously described (Fig. 2a). The 5-OH position of 2 is available for phosphorylation, allowing virtually any protected phosphate to be introduced. However, none of the existing protecting groups are compatible with the subsequent introduction of the coumarin cage. Such protecting groups would need to be stable under acidic and basic conditions and must enable double deprotection under very mild conditions. To meet these stringent requirements, a new phosphate-protecting group is required. The approach described herein is based on an Umpolung strategy that had been exploited in prodrug design for nucleotides. Conceptually, this strategy is useful to generally couple phenol or alcohol protecting groups to phosphates via a benzyl adaptor, greatly enhancing the available protecting group. After coupling P-amidite and oxidation, all protecting groups were then cleaved with piperidine resulting in the dodeca-piperidinium salt. The piperidinium ions can also be exchanged with sodium ions by precipitation. In addition, natural 10 can be prepared following the same strategy by using (Fm)$_2$-P-amidite 5 in the anhydride formation (8% overall yield from 1). This eight-step synthesis represents a general strategy to access 5-InsP$_7$ and caged analogues in scalable amounts (30 mg of 9 have been prepared) and very high quality...
Figure 2 | Synthesis of photocaged 5-InsP₇ and mechanism of LevB cleavage. (a) Synthesis of DEACM 5–InsP₇, 9 and 5–InsP₇, 10 based on fluorenylmethyl (Fm) protection and a novel phosphate-protecting group (LevB). DCI, 4,5-dicyanoimidazole; DEACM, [7-(diethylamino)-coumarin-4-yl]methyl; Fm, fluorenylmethyl; mCPBA, metachloro perbenzoic acid; TFA, trifluoroacetic acid. (b) An adaptor strategy for phosphate release: hydrazine triggers levulinate (red) cleavage and 1,6-elimination (blue) to release free phosphate. Generally, levulinate could also be replaced with other phenol protecting groups.

without the need for a final hydrogenation under aqueous conditions.

**In vitro stability and photophysical properties.** To serve as a useful tool, DEACM 5–InsP₇, 9 must be stable towards enzymatic digestion to enable cellular uptake and release only on photolysis. To test its stability, 9 was incubated in tissue homogenate (brain, liver, Supplementary Fig. 3 and Supplementary Methods) and cell extract (Supplementary Figs 4–5 and Supplementary Methods). Readout was achieved by resolution on polyacrylamide gels (35%, Fig. 3 and Supplementary Methods) and cell extract (Supplementary Figs 4–5 and Supplementary Methods) as verified by PAGE (Fig. 3a, Lanes VI and VII and Supplementary Fig. 4) and HPLC analysis (Supplementary Fig. 6) with 10 as a standard.

Next, the photophysical properties of DEACM 5–InsP₇, 9 were characterized. The quantum yield for the disappearance of 9 decomposed under these conditions over incubation times up to 5 h (Fig. 3a, Lanes III–V and Supplementary Figs 3–5). Thus, 9 is a probe that has the potential to be broadly applied in different cell and tissue types. Importantly, on exposure to ultraviolet light (366 nm, 4 W, distance 10 cm) in extracts, it was cleanly converted into 5-InsP₇, 10, as verified by PAGE (Fig. 3a, Lanes VI and VII and Supplementary Figs 3–4) and HPLC analysis (Supplementary Fig. 6) with 10 as a standard.

**Cellular delivery and uncaging.** Notwithstanding the efficiency of this synthesis, it was found as expected that DEACM 5–InsP₇, 9, like other polyanions, does not readily cross the non-polar membrane of a cell (Fig. 4b,c). To address this problem, its non-covalent complexation, cell uptake and release using guanidinium-rich molecular transporters were studied. 9 was mixed with amphipathic, guanidinium-rich transporter (2,3-Dioleoylphosphatidylethanolamine (DOPE), Lipofectamine 2000 do efficiently deliver 9 into cells (Fig. 4b).
**In vitro and in cellulo release of 5-InsP7.** (a) Analysis of DEACM 5-InsP7, 9 by gel electrophoresis (PAGE) and toluidine blue staining. 9 is stable for hours in rat brain extract (lanes III–V) and can be uncaged by ultraviolet irradiation (lane VI). Lane I: poly-P marker. Lane II: empty. Lane III: 9 in brain extract (3 h). Lane IV: 9 in brain extract (2 h). Lane V: 9 in brain extract (1 h). Lane VI: 9 in brain extract (1 h), then ultraviolet irradiation (15 min). Lane VII: 9 in distilled water, then ultraviolet irradiation (15 min). Lane VIII: 9. Lane IX: 5-InsP7. 10. (b) Analysis of cellular uptake and in cellulo photouncaging with and without MoTr 11 after TiO2 microsphere extraction followed by gel electrophoresis (PAGE). Bands containing 9 and 10 were additionally extracted and analysed by MALDI mass spectrometry. 9 only enters cells in the presence of MoTr 11 (lanes VI, VII) and can be uncaged in living cells (lane VIII). Lane I: Poly-P marker to assess quality of separation. Lane II: empty. Lane III: HeLa cells (control). Lane IV: HeLa cells + 11 (control). Lane V: HeLa cells + 9 (5 h). Lane VI: HeLa cells + 9 + 11 (5 h). Lane VII: HeLa cells + 9 + 11 (16 h). Lane VIII: HeLa cells + 9 + 11 (16 h, then 10 min irradiation 366 nm, 4W). Lane IX: DEACM 5-InsP7, 9 (control). Lane X: 5-InsP7, 10 (control). Lane XI: InsP6 (control).

**Figure 4** | Intracellular delivery of photocaged 5-InsP7 to HeLa cells with a guanidinium-rich transporter. (a) Structure of amphipathic oligocarbonate transporter, 11. (b) Cellular uptake of 9 as determined by flow cytometry. Complexes were formulated at a 1:1 mole ratio of 9 (5 µM) to 11. Values reported are normalized to the autofluorescence of untreated cells. (c) Histogram plot of intracellular fluorescence demonstrates > 99% delivery efficiency to cells.

Delivery and intracellular distribution of 9 were further analysed in HeLa cells by confocal microscopy after 4 and 16 h (Fig. 5). The z-stack analysis shows DEACM 5–InsP7 9 distributed throughout the cytoplasm at both time points (Fig. 5, single z-slice shown). Both diffuse fluorescence and fluorescent puncta are observed, consistent with mixed diffusion or endosomal uptake and release. This is additionally supported by a 65% reduction in cellular uptake when cells were treated at 4°C, a condition known to inhibit most endocytotic processes (Supplementary Fig. 11).

Cellular uptake, stability and efficient uncaging in living cells was additionally verified by extraction of diphospho-inositol polyphosphates and other cellular phosphates based on a recently published TiO2 microsphere enrichment method. Here it is shown that this method can also be used to extract analogues such as 9 from complex cell and tissue lysates (Fig. 3b and Supplementary Methods) enabling studies concerning its intracellular fate after delivery. After incubation of DEACM 5–InsP7 9 with HeLa cells in the presence or absence of MoTr 11 and repeated washings to remove external 9, the extracts prepared from those cells (1 million cells) clearly showed a distinct novel band corresponding to 9 in the PAGE analysis after enrichment with TiO2 and elution (Fig. 3b, lanes VI–VIII), whereas no such uptake could be detected in the control experiment without transporter (Fig. 3b, lane V). To verify its identity, the band corresponding to DEACM 5–InsP7 9 was extracted from the gel and analysed by MALDI mass spectrometry, demonstrating its intracellular stability (Supplementary Fig. 12 and Supplementary Methods) for multiple hours. Moreover, efficient intracellular uncaging by irradiation at 366 nm was proven using the same extraction and resolution method (TiO2 enrichment, then PAGE) in combination with mass spectrometry after extraction of Lane VIII (Fig. 3b). These conditions were found to be of no immediate toxicity (Supplementary Fig. 13 and Supplementary Methods). In summary, the photocaged molecule 9 is efficiently taken up by cells in the presence of MoTr 11, evenly distributed throughout the cytoplasm, stable for multiple hours in its caged form and can be selectively uncaged to 5-InsP7, 10, thus fulfilling the stringent requirements imposed on an intracellular signalling probe.

**PH-domain translocation.** To determine the suitability of the combined delivery and uncaging strategy for a deeper understanding of the effect of InsP7 fluctuations, PH-domain translocation on cytoplasmic InsP7 release was studied. The rationale for this experiment is provided by the lean phenotype displayed by IP6K1 knockout mice on high-fat diet and the observation that...
InsP7 inhibit Akt phosphorylation in vitro and in vivo by binding to the PH-domain9. Collectively, these findings suggest an effect of 5-InsP7 on membrane localization of Akt. However, no tool to augment any InsP7 within seconds in living cells was previously available. With the new tools in hand, HeLa cells were transiently transfected with a plasmid expressing the PH-domain of Akt fused to an enhanced green fluorescent reporter protein (eGFP)48,49. Cells were serum-starved to induce cytoplasmic localization of the PH-domain due to absence of growth factors and therefore inactivation of the PI3K/Akt/mTOR pathway50. PH–eGFP plasma membrane association was then efficiently induced within 10 min on external addition of a combination of growth factors (insulin-like growth factor (IGF); endothelial growth factor (EGF)) into the medium. During the starvation period, cells were loaded with caged InsP79/MoTr11 nanoparticles for 4 h. This treatment alone had no effect on PH-domain localization. Next, cells were irradiated under a confocal laser-scanning microscope with short laser pulses (375 nm, 10 MHz, 30 s) in different areas (Fig. 6 dotted circle, and Supplementary Figs 14–23), and PH-domain localization was traced using the green channel. After photouncaging, a delayed but complete PH-domain translocation from the plasma membrane into the cytoplasm was observed, and these results were repeated several times (n = 4). Significantly, translocation did not occur when cells were incubated with photocaged InsP79 or MoTr11 only (Supplementary Figs 18–23). In these cases, the PH-domains remained localized on the membrane for several hours, demonstrating the need for the presence of all components and ruling out photobleaching of eGFP in the irradiated areas. A detailed analysis of additional micrographs in pseudo-colour with ratiometric changes is shown in the Supplementary Information (Supplementary Figs 14–23). This is the first example of controlled 5-InsP710 augmentation inside of a living cell within a few seconds timeframe coupled to a microscopic readout on the single cell level. We posit that this strategy will be useful to understand InsP7 signalling in more detail as previously possible, as evidenced by the delayed PH-domain translocation observed for the first time in our experiments.

**Discussion**

This study provides a new strategy to synthesize InsP7 that enables introduction of caging subunits. The potential utility of
Photocaged 5-InsP₇ ⁹ was demonstrated by photon-triggered uncaging in rat brain homogenate and other cell extracts. A complex of ⁹ with molecular transporter ¹¹ was then shown to efficiently enter cells after non-covalent nanoparticle assembly. Collectively, these results provide the first example of the synthesis of a photocaged analogue of InsP₇ and of its subsequent delivery into cells using non-covalent complexation with a guanidinium-rich molecular transporter. A recently developed

Figure 6 | PH-domain translocation in irradiated and control cells. Confocal fluorescence microscopy analysis of PH–eGFP translocation in HeLa Kyoto cells after photouncaging in defined areas (dotted circle). (A) Serum-starved cells were loaded with 5 µM ⁹ + ¹¹ for 4 h and then stimulated with IGF and EGF (100 ng ml⁻¹). Robust recruitment of the PH-domain to the membrane is observed. Photouncaging in the dotted area (white circle) is achieved by short ultraviolet laser pulses and the change of fluorescence intensity followed over time (0, 5 and 15 min). (B) Development of the fluorescence intensity (indicated as gray value) over time (0, 5, 15 min) in three different membrane sections (a, b, c; distance in µm). Photouncaging leads to translocation of the PH–eGFP construct into the cytoplasm after 5 min from the membrane of the irradiated cell. After 15 min, complete translocation of the PH-domain into the cytoplasm is observed (B, b and c), whereas in the non-irradiated control cell the PH–eGFP construct remains localized on the membrane (B, a). Images are presented in pseudo-colour, normalized over time. Intensities were acquired pre-saturated, with the entire dynamic range of intensity available. Scale bars, 5 µM.
TiO₂ microsphere enrichment method was applied to study the in cellulo stability of 5-InsP₇ analogues and their efficient photochemical release in combination with MALDI mass spectrometry. We expect that this combined synthesis, delivery and analytical strategy will find widespread and general application in cell signalling studies as a convenient way to rapidly augment 5-InsP₇ with spatiotemporal resolution. Along these lines, it was shown that cytoplasmic release of 5-InsP₇ triggers delayed but complete membrane desorption of the PH-domain of Akt within 15 min. In the human protonecrotic, PH-domains are the 11th most common domain¹, and the new approach described in this publication will enable a systematic understanding of the effect of inositol-pyrophosphate augmentation on protein localization.

**Methods**

**Experimental data of synthetic compounds.** For ¹H, ¹³C and ³¹P NMR spectra of compounds and MALDI and HR-ESI MS spectra see Supplementary Figs 24–70. ¹H NMR spectra were recorded on Bruker 400 MHz spectrometers or Bruker 500 MHz spectrometers (equipped with a cryo platform) at 298 K in the indicated deuterated solvent. ¹³C⁵¹H NMR spectra and ³¹P NMR spectra were recorded with ¹H-decoupling or ¹H coupling on Bruker 162 MHz or Bruker 202 MHz spectrometers (equipped with a cryo platform) at 298 K for the indicated deuterated solvent. All signals were referenced to an internal standard (PPP). ¹³C⁵¹H NMR spectra were recorded with ¹H-decoupling on Bruker 101 MHz or Bruker 125 MHz spectrometers (equipped with a cryo platform) at 298 K in the indicated deuterated solvent. All signals were referenced to the internal solvent signal as standard (CDCl₃, δ 77.0; CDOD, δ 49.0; DMSO-d₆, δ 39.5).

**Detailed synthetic procedures for all new compounds are provided in the Supplementary Information (see Supplementary Methods, chemical synthesis).**

**Cellular uptake by flow cytometry.** Caged-IP₇-9 and oligomer 11 were brought up in pH 7.4 PBS buffer at mM concentrations. HeLa cells were seeded at 40,000 cells per well in a 24-well plate and allowed to adhere overnight. The IP₇-co-oligomer complexes were formed at a 1:1 molar ratio by mixing 8 µl of 1 mM oligomer stock with 8 µl of 1 mM caged-IP₇-9 stock in 184 µl PBS pH 7.4. For conditions with caged-IP₇-9 alone, 8 µl of 1 mM caged-IP₇-9 stock was added to 192 µl PBS pH 7.4. The complexes were allowed to incubate for 30 min at room temperature. The Lipofectamine 2000 control was prepared in OptiMEM according to the manufacturer’s instructions (0.75 µl Lipofectamine into 62.5 µl OptiMEM, 8 µl caged-IP₇-9 stock into 54.5 µl OptiMEM). The cells were washed with ~0.5 ml serum-free DMEM medium, then 400 µl serum-free DMEM was added to wells with untreated cells; 368.75 µl Lipofectamine into 62.5 µl OptiMEM, 8 µl caged-IP₇-9 stock into 54.5 µl OptiMEM). The cells were incubated at 37°C, 5% CO₂ for 16 h. Cells were then washed with 250 µl serum-free DMEM (-FBS) and incubated for 14–16 h at 37°C (5% CO₂).

**After overnight starvation, the cells were washed with PBS (250 µl) and 230 µl DMEM (-FBS) were added. Afterwards, 20 µl sample mixture (consisting of caged InsP₇-Co-oligomer DG7-7 complexes) were added to each well and distributed equally. The final concentration of the complex was 5 µM for each well. Cells were then incubated at 37°C (5% CO₂) for 4 h.**

**Imaging of Akt-PH translocation.** For 4-h incubation with 9 and 11, HeLa cells were washed with 250 µl (2×) imaging buffer (115 mM NaCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM K₂HPO₄, 20 mM HEPES). About 200 µl imaging buffer were added to the cells. Translocation of eGFP-Akt-PH to the membrane was monitored by adding a mixture of growth factors EGF and IGF (100 ng/ml each). Translocation was monitored over 10 min at 37°C (5% CO₂).

**Cells that had responded to treatment with the growth factors and that had the PH-domain localized at the membrane were used in the uncaging experiments by confocal laser-scanning microscopy.** Non-illuminated neighboring cells were used as controls.

**Confocal laser-scanning microscopy.** Imaging was performed on an Olympus IX83 confocal laser-scanning microscope at 37°C in a 5% CO₂ humidified atmosphere (EMBL incubation box). Imaging was performed using an Olympus Plan-APON ×60 (numerical aperture 1.4, oil) objective. The images were acquired utilizing a Hamamatsu C9100-50 EM CCD camera. Image acquisition was performed via Fluoview imaging software, version 4.2. The green channel was imaged using the 488 nm laser line (120 mW cm⁻²) at 7% laser power and a 525/50 nm emission filter. The red channel was imaged using the 559 nm laser (120 mW cm⁻²) at 2.0% laser power and a 643/50nm emission filter. A pulsed 375 nm laser line (10 MHz) was applied for uncaging experiments. For uncaging experiments, circular regions of interest of 4–10 µm diameter were pre-defined. Pre-activation images were captured for five frames (3 s per frame), followed by 30 s of activation within the regions of interest. Recovery images were captured for 35 min at a frame rate of 5 s per frame.

**Image analysis.** Image analysis was conducted utilizing Fiji open source image analysis software tool²,³. Lookup tables were applied to match the colour within the recorded image with the wavelengths of detected light. For comparability, the lookup tables of pre- and postactivated images were set the same weighting.

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Author contributions

LP, D.T.T., R.C.C., I.R.Y., C.J.M., P.A. and S.H. conducted the experiments. H.J.J., P.A.W., C.S., L.B. and G.G. planned the experiments. H.J.J., P.A.W., I.R.V. and C.J.M. wrote the manuscript. All authors discussed the results and revised the manuscript.

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