A Novel Fluorescent Cell Membrane Permeable Caged cyclic ADP-Ribose Analogue*

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Background: The available agonists for cADPR, an endogenous Ca2+ mobilizing nucleotide, are either weak or not cell permeant.

Results: We synthesized a coumarin caged isopropylidene protected cIDPRE (Co-i-cIDPRE) which is a potent and cell permeant cADPR agonist.

Conclusion: Uncaging of Co-i-cIDPRE activates RyRs for Ca2+ mobilization and triggers Ca2+ influx via TRPM2.

Significance: Co-i-cIDPRE should provide a valuable tool to study cADPR/Ca2+ signaling.

Cyclic adenosine diphosphate ribose (cADPR) is an endogenous Ca2+ mobilizer involved in diverse cellular processes. A cell membrane permeable cADPR analogue, cyclic inosine diphosphoribose ether (cIDPRE), can induce Ca2+ increase in intact human Jurkat T-lymphocytes. Here we synthesized a coumarin caged analogue of cIDPRE (Co-i-cIDPRE), aiming to have a precisely temporal and spatial control of bioactive cIDPRE release inside the cell using UV uncaging. We showed that Co-i-cIDPRE accumulated inside Jurkat cells quickly and efficiently. Uncaging of Co-i-cIDPRE evoked Ca2+ release from endoplasmic reticulum (ER), with concomitant Ca2+ influx in Jurkat cells. Ca2+ release evoked by uncaged Co-i-cIDPRE was blocked by knockdown of ryanodine receptors (RyR) 2 & 3 in Jurkat cells. The associated Ca2+ influx, on the other hand, was abolished by double knockdown of Stim1 and TRPM2 in Jurkat cells. Furthermore, Ca2+ release or influx evoked by uncaged Co-i-cIDPRE was recapitulated in HEK293 cells that overexpress RyRs or TRPM2, respectively, but not in wildtype cells lacking these channels. In summary, our results indicate that uncaging of Co-i-cIDPRE incites Ca2+ release from ER via RyRs and triggers Ca2+ influx via TRPM2.

Cyclic adenosine diphosphate ribose (cADPR) is an endogenous second messenger that mobilizes Ca2+ release in a wide variety of cell types and species. cADPR is formed from nicotinamide adenine dinucleotide (NAD) by ADP-ribosyl cyclases. CD38 is the main mammalian ADP-ribosyl cyclase. cADPR elicits Ca2+ release from the endoplasmic reticulum (ER) through the ryanodine receptors (RyRs), and can also modulate the transient receptor potential cation channel subfamily M member 2 (TRPM2) for Ca2+ influx (1-4). Although all three RyR isoforms have been shown to mediate cADPR-induced Ca2+ release (1-3), evidence regarding whether cADPR acts directly on the receptors is lacking (5). Instead, two cADPR-binding proteins, 140- and 100-Kda proteins, have been identified in sea urchin egg homogenates by using 8-N3-cADPR, a cADPR antagonist, as a photoaffinity probe (6). Also, both calmodulin and FK506 binding protein (FKBP) have been shown to be required for
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cADPR action (1-3,7-9). These data suggest that cADPR does not directly bind to the ryanodine receptors, but acts through some intermediate proteins, whose definitive identities remain to be established.

Given the important physiological role of cADPR, a number of cADPR analogues have been generated chemically or from the corresponding NAD analogues using ADP-ribosyl cyclase (10-13). The first successful total chemical synthesis of cADPR and its analogues was not reported until 2000 by Shuto et al (14), despite repeated attempts right after cADPR was discovered. In the Shuto method, a large group is introduced to the 8-position of purine to force the nucleoside to adopt the syn conformation, such that the subsequent intra-molecular cyclization could be achieved. A later improvement, using an activated precursor, succeeded in intra-molecular pyrophosphate formation even without a large substituent at the 8-position (14). The success of chemical synthesis has promoted the synthesis of a number of cADPR analogues that could not be synthesized by the enzymatic method in recent years (15-23). These analogues can be grouped into two categories: either derivatives of cyclic IDP-Ribose (cIDPR) or cyclic ADP-carbocyclic-Ribose (cADPcR). These cADPR analogues have been used to elucidate some important structural and functional properties of cADPR (13).

Like all other cytosolic messengers, cADPR is hydrophilic and cannot cross the plasma membrane. Therefore, cell-permeant cADPR analogues are valuable research tools in dissecting the mechanism of cADPR-induced Ca$^{2+}$ release. A number of cADPR analogues with modification at the N-1 position have been synthesized by us, such as those using an ether linkage to substitute for the ribose of cIDPR (16-23). These mimics not only retain the Ca$^{2+}$ releasing activity, but more importantly, are also membrane permeant. A moderate agonistic analog of cADPR is obtained after both northern and southern riboses are substituted with ether linkages (19). More recently, the nucleobase of cADPR has been simplified and a novel cADPR analogue, cTDPRE, is synthesized using the click chemistry, and it is biologically active in human Jurkat T cells (22,24). Yet, the main drawback for these cADPR agonists is that they are not particularly potent. Here we synthesized a novel fluorescent caged cADPR analogue, coumarin caged isopropylidene protected cIDPRE (Co-i-cIDPRE), and found that it is a potent and controllable cell permeant cADPR analogue. Moreover, we demonstrated that uncaging of Co-i-cIDPRE activates RyRs for Ca$^{2+}$ mobilization and triggers Ca$^{2+}$ influx via TRPM2.

EXPERIMENTAL PROCEDURES

Chemistry- All of the chemical reagents used were purchased from Sigma. Compound 1 was synthesized using the method as described previously (25). Phosphorylation of compound 1 was then performed (26). Briefly, compound 1 (40 mg, 0.1 mmol) was dissolved in a solution of 1H-tetrazole (70 mg, 1 mmol) in CH$_3$CN (2 ml) with argon protection and was stirred for 15 min at room temperature. Next, dibenzyl N,N-diisopropylphosphoramidite (142 mg, 0.6 mmol) was added dropwise over 1 min. After stirring for overnight, the reaction mixture was cooled to 0 °C and tert-Butyl hydroperoxide (0.18 ml of 5.5 M solution in decane) was added. After another 4h of the reaction exposed to air, this phosphorylation procedure was finished. Flash chromatography was then used to purify compound 2 (70 mg, 75% yield). Hereafter, compound 2 (50 mg, 0.055 mmol) was suspended with 10% Pd/C (20 mg) in CH$_3$OH (5 ml) and the suspension was stirred at room temperature under H$_2$ (0.4 atm) for 1h for debenzylation. The catalyst was subsequently filtered off, and the filtrate was evaporated. The dried filtrate was dissolved in dry DMSO with 1,1'-carbonyldiimidazole (CDI) (45 mg, 0.28 mmol) and underwent a microwave cyclization at 85°C for 1h. Afterwards, compound 3 (45 mg, 40% yield), as a triethylamine salt, was purified by HPLC on a C18 reversed phase column, eluting with a linear gradient of 0–60% CH$_3$CN in TEAA buffer (0.05 M, pH 7.5). Compound 3 was subsequently changed to tetrabutylammonium hydroxide. After lyophilization, compound
3 tetrabutylamine salt (12 mg) was dissolved in anhydrous CH$_3$CN (2 ml), and mixed with 4-(bromomethyl)-7-acetoxy-coumarin (18 mg, 0.06 mmol). This mixture was allowed reflux under 85°C for 5 h. Finally, the target compound 4 (3.3 mg, 30% yield) was purified by HPLC on a C18 reversed phase column, eluting with a linear gradient of 0–80% CH$_3$CN in TEA buffer (0.05 M, pH 7.5) (Figure 1). According to the $^1$H NMR spectrum, this caged structure represented a mixture of more than one mono-caged isomers (Figure S1). They all could be efficiently photolyzed into $i$-cIDPRE under the UV flash as detected by HPLC analysis (Figure 2B). $^1$H NMR (500 MHz, D$_2$O) δ 8.4, 8.1 (s, each 1 H), 8.0-6.0 (m, 4 H), 5.90 (m, 1 H), 5.28 (m, 1 H), 4.42 (m, 1 H), 4.1-3.4 (m, 11 H), 2.3-2.1 (m, 3 H, -COCH$_3$), 1.55, 1.38 (each s, each 3 H), all of the other peaks were subjected to the signals from tetrabutylamine salt. $^3$P NMR (121 MHz, D$_2$O) δ -10.30, -10.56 ppm. HRMS (ESI, positive) for C$_{33}$H$_{45}$O$_6$P$_2$, Calcd. 755.1361 [M+1]$^+$, found 755.1359 (Figure S1).

Cell culture-The human Jurkat T lymphocytes and human embryonic kidney (HEK) 293 cells were obtained from ATCC (Manassas, USA). HEK293 cells that overexpress RyR2 or RyR3 were kindly provided by Dr. King-Ho Cheung (the University of Hong Kong). Jurkat cells were normally cultured in RPMI Medium 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin, and 2 mM Hepes Buffer (pH 7.4) at 5% CO$_2$ and 37°C. HEK293 cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS and 100 units/ml penicillin/streptomycin at 5% CO$_2$ and 37°C.

Western blot analysis-Western blot analysis was performed as described previously (27). Briefly, cells were lysed in an ice-cold EBC lysis buffer (50 mM HEPESS at pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 150 µM PMSF, 10 mM NaF, 10 ng/ml leupeptin, 1 mM DTT, and 1 mM sodium vanadate) and passed through a 21-gauge needle several times to disperse any large aggregates. Protein concentrations of the cell lysates were determined by Bradford protein assay. 30 µg of protein per lane was diluted in the standard SDS-sample buffer and subjected to electrophoresis on 8 or 10% SDS polyacrylamide gels. Proteins were then transferred to an Immobilon PVDF membrane (Millipore, Billerica, MA), blocked with 5% milk in TBST (20 mM Tris, 150 mM NaCl, pH 7.6), and incubated with the primary antibody (RyRs, SC-13942, Santa Cruz Biotechnology, 1:500 dilution; Stim1, #610954, BD Biosciences, 1:1000 dilution; TRPM2, SC-19198, Santa Cruz Biotechnology, 1:500 dilution) overnight. After washing with TBST, the blots were probed with a secondary antibody (1:3000 dilution) for detection by chemiluminescence.

Ryr2, Ryr3, TRPM2, and Stim1 shRNA lentivirus production and infection-The optimal 21-mers were selected in the human ryr2, ryr3, trmp2, and stim1 genes (Table S1). One 21-mer was selected in the GFP gene as a control. These sequences were then cloned into pLKO.1 vector for expressing shRNA. The shRNA lentivirus production was performed in 293T cells as described previously (28). For infection, Jurkat cells were plated at a density of 3×10$^5$ cells/well in 6-well plates. On the next day, 100 µL pools of shRNAs lentivirus were added to the cells in fresh medium containing 8 µg/mL polybrene. Two days later, cells were selected in fresh medium containing puromycin (3 µg/mL) for 3–5 days. The puromycin-resistant cells were pooled and the knockdown efficiency was verified by both quantitative real-time RT-PCR and/or Western blot analyses. The #1 TRPM2 shRNA was used for the double knockdown with Stim1.

Quantitative real-time RT-PCR analysis-The quantitative real time RT-PCR using the iScript™ One-Step Kit with SYBR® Green (Invitrogen) was performed normally in Bio-Rad MiniOpticon™ Real-time PCR Detection System according to the manufacturer’s instructions. The primers for detecting ryr2 or ryr3 mRNAs are listed in Table S1.

Transient transfection- HEK293 cells were plated at a density of 3×10$^5$ cells/well in 6-well plates. On the next day, 2 hours before transfection, the medium was changed to an antibiotics-free medium. The pCI-CFP-hTRPM2 or empty vector
pCl-CFP was then transfected into cells by Lipofectamine™ 2000 (Invitrogen). 24 hours after transfection, the medium was changed to regular medium, and TRPM2-CFP or CFP positive cells were finally used for Ca\(^{2+}\) measurement after another 24 hours. 

**Ca\(^{2+}\) measurement**-Ca\(^{2+}\) measurement was performed as described previously (29). Briefly, Jurkat cells (2×10^5 cells/well) or HEK293 cells (6×10^5 cells/well) were plated in 24-well plate coated with 100 µg/mL or 10 µg/mL Poly L-lysine (Sigma, P6282), respectively. Both cells were incubated first in serum-free medium overnight for adherence before changing to regular medium. The adherent cells were incubated with 2 µM Fluo-4 AM (Invitrogen) in Hanks’ Balanced Salt Solution (HBSS) with or without calcium for 30 min in the dark at 37 °C. The cells were then washed with HBSS twice and incubated in 200 µL HBSS. Thereafter, the cells were put on the stage of an Olympus inverted epifluorescence microscope and incubated with or without caged compound for 5 min, followed by UV (370 nm) flash for 1 second, which was repeated every 7 seconds during the measurement of fluorescence intensity at 480 nm using a 20X objective. Images were collected by a CCD camera every 7 seconds and analyzed by a Cell R imaging software. For Ca\(^{2+}\) mobilization in single cell, a 60X oil immersion objective was used.

**Data Analysis**-In each measurement, intracellular Ca\(^{2+}\) concentration was calculated using the formula, 

\[ [Ca^{2+}] = \frac{K_d(F-F_{\text{min}})}{F_{\text{max}}-F} \]

\(K_d = 345 \text{ nM}\), if the value fit within the indicating ranges for Fluo-4. \(F_{\text{max}}\) was determined by exposing cells to 10 mM Ca\(^{2+}\) and 5 µM Ionomycin, and \(F_{\text{min}}\) was determined by addition of 4 mM EGTA and 5 µM Ionomycin to cells. For Ca\(^{2+}\) concentrations that go beyond the Fluo-4 indicating range, \(F/F_0\) (\(F_0\): fluorescence intensity at the start point of measurement) were used instead. All of the data were averaged from at least three independent experiments. Significant differences of peak Ca\(^{2+}\) level and rise time for maximum Ca\(^{2+}\) concentration between groups were determined by the Student’s t test, in which \(p<0.05\) was validated to be significant.

**Permeability kinetics**-Jurkat cells were plated in 24-well plates as described above. The cells were then incubated with 200 µM Co-i-cIDPRE for up to 5 min. Thereafter, cells were washed with regular HBSS medium and incubated in medium without Co-i-cIDPRE for another 25 min. At the indicated time points, cells at three individual wells were washed again with HBSS and subjected to a reading of the fluorescence intensity at 337 nm.

**RESULTS**

**Design and synthesis of a new caged isopropylidene protected cADPR analogue:** *Coumarin caged cIDPRE (Co-i-cIDPRE)*. Many derivatives of cADPR have been synthesized enzymatically or chemically (13). Some of these cADPR mimics are strong cADPR agonists, but like cADPR itself, are cell impermeant, such as 3-deaza-cADPR (30). Others are cell-permeant, but are weak agonists of cADPR, such as cIDP-DE and cIDPRE (18,19). In addition, almost all of these cADPR agonists are difficult to synthesize and some of them are unstable. Among them, cIDPRE is a relative stable and permeable analogue of cADPR, and we have been able to simplify the synthesis route of cIDPRE from the original 8 steps (25) to 5 steps with good yield (Figure 1A). Two important improvements were used. Firstly, dibenzyl N,N-diisopropylphosphoramidite was used in the phosphorylation step, which allowed us to carry out intra-molecular cyclization right after debenzylation by hydrogenation without further HPLC purification. Secondly, intra-molecular cyclization was achieved by CDI catalysis under a microwave condition, which allowed using high concentration of reagents and resulted in a shorter reaction time and higher yield. In the process of simplifying the synthesis of cIDPRE, we found that the isopropylidene protected cIDPRE (i-cIDPRE) was more stable than cIDPRE. In addition, i-cIDPRE evoked a Ca\(^{2+}\) increase in Jurkat cells at a similar potency as cIDPRE (Figure S2).

To improve the biological activity of i-cIDPRE, we reasoned that adding a caged group to one of the phosphates on i-cIDPRE
could increase its membrane permeability and enable it to accumulate inside cells without mobilizing Ca\(^{2+}\). Photolysis by UV can then release the bioactive cIDPRE, providing more precise control of its Ca\(^{2+}\)-signaling function. We chose coumarin as the caged group not only because of its lipophilicity that can enhance membrane permeability, but also because it is fluorescent, which can facilitate the monitoring of its permeability kinetics. To further increase lipid-solubility and stability of the caged compound (31), the 7-OH on the coumarin was acetylated. The synthesis of the coumarin caged i-cIDPRE (Co-i-cIDPRE) is shown in Figure 1B. Characterizations of the compound using \(^1\)H NMR, \(^3\)P NMR and high resolution mass spectrometry are consistent with the predicted structure (Figure S1). It is also noted that the final Co-i-cIDPRE fraction collected during HPLC purification contains a mixture of isomers that were caged at different phosphates of i-cIDPRE and/or with different conformations, although only one caged group existed in it.

We found that Co-i-cIDPRE was relatively stable, with only 17.6% hydrolyzed to i-cIDPRE after storage in its lyophilized form at –20°C in the dark for 240 days (data not shown). The fluorescence spectrum of Co-i-cIDPRE is shown in Figure 2A, with an excitation maximum at 337 nm and an emission maximum at 475 nm. After continuous UV illumination for 10 min, Co-i-cIDPRE was almost completely photolyzed into bioactive i-cIDPRE and coumarin (Figure 2B). The permeation and efflux kinetics of Co-i-cIDPRE in Jurkat cell was also analyzed. As shown in Figure 2C, the fluorescent Co-i-cIDPRE accumulated inside Jurkat cells quickly within 3 min of incubation, and reached saturation within 5 min. After Co-i-cIDPRE was removed from the medium at 5 min, the fluorescent intensity of cells was only slowly decreased over time, indicating the efflux rate of Co-i-cIDPRE is small. The image of Jurkat cells loaded with the fluorescent Co-i-cIDPRE is shown in Figure 2D, and the quantitative analysis of the influx and efflux of Co-i-cIDPRE is shown in Figure S3.

**Pharmacological characterization of Co-i-cIDPRE.** Although Co-i-cIDPRE is fluorescent, no interference was observed when using either Fluo-4 or Fluo-3 to monitor its Ca\(^{2+}\)-mobilizing property. As expected, without UV uncaging, Co-i-cIDPRE did not evoke any Ca\(^{2+}\) changes in Jurkat cells. Subsequent UV flashes evoked Ca\(^{2+}\) increases that were much higher than that induced by either cIDPRE itself or by photolyzing another caged analogue of cADPR, NPE-cADPR, at the same concentration (Figure 3A and Figure S4). Controls showed that, in cells without the Ca\(^{2+}\) indicator, uncaging of Co-i-cIDPRE did not produce any fluorescence changes, indicating that the signals indeed reflected Ca\(^{2+}\) changes (data not shown). Removal of external Co-i-cIDPRE by washing cells with regular HBSS buffer altered neither the rise time nor the amplitude of the Ca\(^{2+}\) increases, indicating it was the photolyzed cIDPRE inside the cells that was responsible for inducing the Ca\(^{2+}\) changes (Figure 3B).

The extent of the photolysis-induced Ca\(^{2+}\) changes was dependent on the concentration of Co-i-cIDPRE in both the presence and absence of extracellular Ca\(^{2+}\), although the Ca\(^{2+}\) increases observed in the presence of extracellular Ca\(^{2+}\) were much higher and sustained, indicating that Ca\(^{2+}\) influx also contributed (Figures 3C, 3D, and S5). Pretreating cells with thapsigargin, a specific SERCA inhibitor, abolished the Ca\(^{2+}\) increase in the absence of external Ca\(^{2+}\), consistent with the fact that cADPR induces Ca\(^{2+}\) release from the ER pools (Figure 3E). In summary, these data demonstrate that UV uncaging of Co-i-cIDPRE induces Ca\(^{2+}\) release from ER pools, accompanied with extracellular Ca\(^{2+}\) influx.

**Requirement of RyRs for uncaged Co-i-cIDPRE-induced Ca\(^{2+}\) release.** Ample evidence indicates cADPR targets the ryanodine receptor on the ER membrane in many cell types (32,33). Indeed, pretreatment with the RyR antagonist, high concentrations of ryanodine, significantly inhibited the Ca\(^{2+}\) increases triggered by uncaging the Co-i-cIDPRE in the Jurkat cells, in both the presence (Figure 4A) and the absence (Figure 4B) of extracellular Ca\(^{2+}\). Both RyR2 and RyR3 were detected in
the Jurkat cells. Thus, single or double knock-down experiments of both types of RyRs in Jurkat cells were done (Figures 4C, 4D, and S6). Consistently, the ability of Co-i-cIDPRE to initiate Ca\(^{2+}\) release after UV flashes was significantly inhibited in these RyRs knockdown cells (Figure 4E). Further support came from using another cell type, HEK293, which stably over-expresses either RyR2 or RyR3. Both cells were shown to be responsive to caffeine (Figure S7) or photo-uncaging of Co-i-cIDPRE (Figure 4F), while the wildtype HEK293 cells lacking the RyRs were non-responsive to either treatment (Figures 4F and S7). In summary, our results definitely demonstrate that photolysis of Co-i-cIDPRE evokes Ca\(^{2+}\) releases via RyRs.

**Requirement of TRPM2 for uncaged Co-i-cIDPRE-triggered Ca\(^{2+}\) influx.** It is conceivable that Ca\(^{2+}\) release from ER induced by uncaged Co-i-cIDPRE leads to partially depleted ER Ca\(^{2+}\) pools, which then activates canonical store-operated Ca\(^{2+}\) channels (SOCs) for Ca\(^{2+}\) influx. To confirm that canonical SOCs are responsible for the sustained phase of the Ca\(^{2+}\) increase induced by uncaged Co-i-cIDPRE, Stim1 was knocked down in Jurkat cells by shRNAs (Table S1 and Figure 5A). The ability of uncaged cIDPRE-induced Ca\(^{2+}\) influx was markedly inhibited, but not abolished, in Stim1 knockdown cells (Figure 5D). Since high concentrations of ryanodine or double knockdown of RyR2&3 also failed to completely block uncaged Co-i-cIDPRE-induced Ca\(^{2+}\) entry (Figures 4A and 4E), we suspected that other Ca\(^{2+}\) channels are also involved. Given that cADPR can modulate TRPM2 for Ca\(^{2+}\) influx (4,34), we examined whether TRPM2 also contributes to uncaged Co-i-cIDPRE-induced Ca\(^{2+}\) influx. The expression of TRPM2 in Jurkat cells was effectively knocked down by a series of shRNAs (Table S1 and Figure 5B). As shown in Figure 5D, in TRPM2-knockdown cells, the sustained phase of the Ca\(^{2+}\) increase induced by photolyzing Co-i-cIDPRE was also markedly inhibited, but not eliminated. Thus, both Stim1 and TRPM2 were knocked down in Jurkat cells (Figure 5C), and Co-i-cIDPRE-induced Ca\(^{2+}\) influx was almost completely blocked in the double-knockdown cells (Figure 5D), in which the pattern of Ca\(^{2+}\) changes now resembled that observed in the absence of extracellular Ca\(^{2+}\) (Figure 3D). These data indicated that both canonical SOCs and TRPM2 contribute to uncaged cIDPRE-induced Ca\(^{2+}\) influx in Jurkat cells. To further assess whether uncaged Co-i-cIDPRE directly activates TRPM2 for Ca\(^{2+}\) influx, a CFP-tagged TRPM2 was transiently expressed in HEK293 cells that lack endogenous TRPM2 channel (Figure 6A). As shown in Figure 6B, uncaging of Co-i-cIDPRE triggered Ca\(^{2+}\) influx only in TRPM2-CFP expressed HEK293 cells, but not in wildtype HEK293 cells lacking the TRPM2 channels. Similar results have been observed in NPE-cADPR treated cells (Figure 6C). Thus, these data clearly demonstrated that uncaging of Co-i-cIDPRE or NPE-cADPR directly activates TRPM2 for Ca\(^{2+}\) influx.

**DISCUSSION**

In this study, we describe the synthesis and characterization of a fluorescent caged analogue of cADPR, Co-i-cIDPRE. We found that Co-i-cIDPRE entered intact human Jurkat T-lymphocytes quickly and accumulated inside cells with small leakage. After UV photolysis, Co-i-cIDPRE induced a much stronger Ca\(^{2+}\) increase than that of cIDPRE itself or NPE-cADPR, the caged cADPR previously synthesized (35). The sources of the Ca\(^{2+}\) increase are from the ER Ca\(^{2+}\) pools, with concomitant Ca\(^{2+}\) influx. The antagonists for RyRs or knockdown of the RyRs in Jurkat cells eliminated uncaged Co-i-cIDPRE-evoked Ca\(^{2+}\) release while knockdown of Stim1 and TRPM2 abolished the concomitant Ca\(^{2+}\) influx. Consistently, in another cell type, HEK293, uncaging of Co-i-cIDPRE only induced Ca\(^{2+}\) release or triggered Ca\(^{2+}\) influx in cells that overexpress RyRs or TRPM2, respectively, but not in wildtype cells lacking these channels. Therefore, Co-i-cIDPRE is a cell permeant cADPR analogue that can mobilize Ca\(^{2+}\) release via RyRs and induce Ca\(^{2+}\) influx via TRPM2 and SOCs.

The parent compound of Co-i-cIDPRE, cIDPRE, has only moderate cell membrane permeability and its synthesis is quite
difficult (18). We simplified the synthesis of cIDPRE by using dibenzyl N,N-diisopropylphosphoramidite for di-phosphorylation and performing cyclization under microwave-assisted condition, which resulted in shorter steps and greater increase in yield. Adding a coumarin caged group to one of the phosphates in i-cIDPRE and acetylation of the 7-OH on the coumarin further increase the liposolubility and stability of the caged compound, thereby facilitating its entry into cells (Figure 2C). During the coumarin caging step, isopropylidene protection on the two active hydroxyl groups in cIDPRE also greatly increased the yield of the caged compound (data not shown). Moreover, we found that the removal of Co-i-cIDPRE in the medium after cell loading had no effects on the Ca\(^{2+}\) increases induced by subsequent uncaging of Co-i-cIDPRE (Figure 3B). These data not only demonstrate that it is the cytosolic, not extracellular, uncaged Co-i-cIDPRE that induces Ca\(^{2+}\) increases, but also is in line with the fact that Co-i-cIDPRE can accumulate inside cells with few leaking out (Figure 2D). We reason that the acetylated group on coumarin-caged compound is hydrolyzed by an esterase once it enters the cells, thereby decreasing the ability of the caged compound to leak out of the cells. Interestingly, we found that NPE-cADPR can also enter slowly into intact Jurkat cells and incite Ca\(^{2+}\) increases after UV photolysis. However, washing out the extra NPE-cADPR in the medium could markedly inhibit the ability of NPE-cADPR to induce Ca\(^{2+}\) increases, in both the rising time and the amplitude of Ca\(^{2+}\) current (Figure S8). Thus, these data suggest that NPE-cADPR enters or exits the cell membrane via concentration gradients and can be removed by washing. Taken together, our data clearly demonstrated that Co-i-cIDPRE has the advantages of being a stable, highly cell permeant, and potent cADPR analogue.

It has been shown that cADPR targets the RyR, but which specific isoform it targets remains to be determined (1-3). Here we showed that both RyR2 and RyR3 are expressed in Jurkat cells. Individual knockdown of these isoforms inhibited cIDPRE or cADPR-induced Ca\(^{2+}\) release to a similar extent, while double knockdown of both isoforms abolished cADPR-induced Ca\(^{2+}\) release in Jurkat cells (Figure 4E). These data indicate that cADPR does not distinguish either isoform. However, it still remains to be determined whether cADPR binds directly to the RyRs or through some unknown proteins. Recently, the long-sought-after store-operated Ca\(^{2+}\) entry proteins were identified using a genome-wide RNAi screen by several groups (36-38). Co-i-cIDPRE could be a valuable tool to be used in a similar cell based screening for cADPR-interacting proteins or regulators.

TRPM2 is a Ca\(^{2+}\) permeable, non-selective cation channel with a unique C-terminal diphosphoribose hydrolase and Nudix-like domain. The main activator for TRPM2 is intracellular ADP-ribose (ADPR) via its C-terminus Nudix-like domain. TRPM2 can also be positively regulated by [Ca\(^{2+}\)]\(i\), cADPR, H\(_2\)O\(_2\), and NAADP, and negatively regulated by AMP and acidic pH. The mechanism for the regulation of TRPM2 by these factors, including cADPR, is not clear (34,39,40). It is generally thought that cADPR can act with ADPR to synergistically activate TRPM2 (34). It has also been proposed that the activating effect of cADPR may be due to its enzymatic conversion to ADPR inside cells, possibly via CD38 (41). Here we found that uncaging either Co-i-cIDPRE (Figure 5D and Figure 6B) or NEP-cADPR (Figure 6C and data not shown) not only activated endogenous TRPM2 in Jurkat cells but also the exogenous expressed TRPM2 in HEK293 cells, producing Ca\(^{2+}\) influx in both cases. Photolysis of Co-i-cIDPRE generates only cIDPRE and coumarin (Figure 2B), not ADPR. Likewise, photolysis of NPE-cADPR produces only cADPR, not ADPR (data not shown). Moreover, CD38 is not expressed in HEK293 cells (data not shown); thus it is less likely that cADPR is converted to ADPR by CD38 in HEK293 cells. In addition, cIDPRE was found to be resistant to CD38’s hydrolytic activity in vitro (17). Taken together, all these data support that cADPR directly activates TRPM2 for Ca\(^{2+}\) influx. Yet, the exact mechanism of how cADPR activates the
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TRPM2 channel remains to be determined.

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FOOTNOTES

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The abbreviations used are: cADPR, cyclic adenosine diphosphate ribose; cIDPRE, \(N^4\)-[(5\''-O-Phosphorylethoxy)methyl]-5\''-O-phosphorylinosine 5\',5\''-cyclicpyrophosphate; Co-i-cIDPRE, coumarin caged isopropylidene protected cIDPRE; TRPM2, transient receptor potential cation channel subfamily M member 2; SOCs, store-operated channels; RyRs, ryanodine receptors; ER, endoplasmic reticulum.

FIGURE LEGEND

Figure 1. Synthesis route of coumarin caged isopropylidene protected cIDPRE (Co-i-cIDPRE). (A) Synthesis of i-cIDPRE. (B) Synthesis of Co-i-cIDPRE.

Figure 2. Physical Characteristics of Co-i-cIDPRE. (A) Fluorescence spectra of Co-i-cIDPRE with \(\lambda_{\text{ex}} = 337\) nm and \(\lambda_{\text{em}} = 475\) nm. (B) HPLC analysis of Co-i-cIDPRE before and after UV
photolysis. (C) The permeation and efflux kinetics of Co-i-cIDPRE in human Jurkat cells. Cells were incubated with Co-i-cIDPRE (200 μM) in HBSS for 5 minutes, thereafter Co-i-cIDPRE was washed away and the cells were incubated in Co-i-cIDPRE free HBSS for another 25 minutes. (D) Fluorescent image of Jurkat cells loaded with Co-i-cIDPRE (200 μM) (20X).

Figure 3. Co-i-cIDPRE evokes Ca²⁺ increase in human Jurkat cells after UV photolysis. (A) Co-i-cIDPRE (200 μM) incited much stronger Ca²⁺ increases after UV photolysis than that by cIDPRE (200 μM) in Jurkat cells. Fluo-4 loaded Cells were incubated in regular HBSS containing extracellular Ca²⁺ during the experiment. (B) After Fluo-4 loaded Jurkat cells were incubated with Co-i-cIDPRE (120 μM) in regular HBSS containing extracellular Ca²⁺, washing away Co-i-cIDPRE in the medium did not have any effect on Ca²⁺ changes triggered by uncaging of Co-i-cIDPRE. (C) and (D) In Fluo-4 loaded Jurkat cells, uncaging of Co-i-cIDPRE triggered Ca²⁺ increases in a dose dependent manner in both the presence (C) and absence of extracellular Ca²⁺ (D). (E) In Fluo-4 loaded Jurkat cells, thapsigargin (1 μM) pretreatment abolished Co-i-cIDPRE (120 μM) induced Ca²⁺ increases in the absence of extracellular Ca²⁺. Data quantifications of [Ca²⁺]p peak induced by drug treatment in (A), (C), (D), and (E) were expressed as mean ± S.E., n=30-40 cells, p < 0.05. In (A), (C), (D), and (E), cells were all continuously incubated with Co-i-cIDPRE throughout the experiments.

Figure 4. The requirement of RyRs for Co-i-cIDPRE-triggered Ca²⁺ releases. (A) and (B) Pretreatment of Fluo-4 loaded Jurkat cells with ryanodine (100 μM) inhibited Co-i-cIDPRE (120 μM)-induced Ca²⁺ increases in both the presence (A) and absence (B) of extracellular Ca²⁺ after UV uncaging. (C) Knockdown of RyR2 or RyR3 in Jurkat cells was verified by real-time RT PCR analysis. (D) Double knockdown of RyR2 and RyR3 in Jurkat cells was confirmed by Western blot analysis with a pan RyR antibody. (E) Knockdown of RyR2, RyR3, or both RyRs 2 & 3 inhibited Co-i-cIDPRE (120 μM)-induced Ca²⁺ increases in Jurkat cells after UV uncaging. Fluo-4 loaded Cells were incubated in regular HBSS containing extracellular Ca²⁺ during the experiment. (F) Uncaging of Co-i-cIDPRE (120 μM) induced Ca²⁺ release only in HEK293 cells that stably express RyR2 or RyR3. HEK293 Cells were loaded with Fluo-4 and incubated in regular HBSS containing extracellular Ca²⁺ during the experiments. Data quantifications of [Ca²⁺]p peak or [Ca²⁺] at 8 minute induced by uncaged Co-i-cIDPRE in (A), (B), (E), and (F) were expressed as mean ± S.E., n=30-40 cells, p < 0.05. In (A), (B), (E), and (F), cells were all continuously incubated with Co-i-cIDPRE throughout the experiments.

Figure 5. The requirement of SOC1s and TRPM2 for Co-i-cIDPRE-triggered Ca²⁺ influx in human Jurkat cells. (A), (B), and (C) Knockdown of Stim1 (A), TRPM2 (B), and both Stim1 and TRPM2 (C) in human Jurkat cells was verified by Western blot analysis with an antibody against Stim1 or TRPM2. (D) Knockdown of Stim1, TRPM2, or both Stim1 and TRPM2 in Jurkat cells inhibited Co-i-cIDPRE-induced Ca²⁺ influx in Fluo-4 loaded cells after UV uncaging. Cells were all continuously incubated with Co-i-cIDPRE (120 μM) in regular HBSS containing extracellular Ca²⁺ throughout the experiments. Data quantifications of [Ca²⁺]p peak or [Ca²⁺] at 8 minute induced by uncaged Co-i-cIDPRE were expressed as mean ± S.E., n=30-40 cells, p < 0.05.

Figure 6. Ca²⁺ influx triggered by Co-i-cIDPRE or NPE-cADPR in TRPM2-CFP expressed HEK293 cells. (A) The DIC and fluorescence images of HEK293 cells that transiently express TRPM2-CFP. (B) and (C) Uncaging of Co-i-cIDPRE (B) or NPE-cADPR (C) only induced Ca²⁺ influx in Fluo-4 loaded HEK293 cells that expresses TRPM2-CFP. Data quantifications of [Ca²⁺]p peak induced by uncaged Co-i-cIDPRE were expressed as mean ± S.E., n=20-30 cells, p < 0.05. Cells were all continuously incubated with Co-i-cIDPRE (120...
μM) or NPE-cADPR (120 μM) in regular HBSS containing extracellular Ca²⁺ throughout the experiments.
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Figure 1

A

\[
\text{Compound 1} \xrightarrow{1. \text{BnO}} \text{Compound 2} \xrightarrow{2. \text{1H-Tetrazole, BuOOH}} \text{Compound 3}
\]

\[
\text{H}_2\text{N/C} \xrightarrow{\text{MeOH, rt, 1h}} \text{CDI} \xrightarrow{\text{DMF, Microwave, 85°C, 1h}} \text{Compound 3}
\]

B

\[
\text{Compound 3} \xrightarrow{\text{MeCN, 85°C, 5h}} \text{Compound 4}
\]
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A novel fluorescent cell permeant caged cADPR analogue
A novel fluorescent cell permeant caged cADPR analogue
A novel fluorescent cell membrane permeable caged cyclic ADP-Ribose analogue
Pei-Lin Yu, Zhe-Hao Zhang, Bai-Xia Hao, Yong-Juan Zhao, Li-He Zhang, Hon-Cheung Lee, Liang-Ren Zhang and Jianbo Yue

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