Synthesis and Biological Evaluation of Novel Membrane-Permeant Cyclic ADP-Ribose Mimics: N^1-[(5″-*O*-Phosphorylethoxy)methyl]-5′-*O*-phosphorylinosine 5′,5″-Cyclicpyrophosphate (cIDPRE) and 8-Substituted Derivatives

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N^1-[(5″-*O*-Phosphorylethoxy)methyl]-5′-*O*-phosphorylinosine 5′,5″-cyclicpyrophosphate (cIDPRE **2a**) and the 8-substituted derivatives 8-bromo-, 8-azido-, 8-amino-, and 8-Cl-cIDPRE (**2b**−**e**) were synthesized from N^1-[(5″-acetoxyethoxy)methyl]-2′,3′-*O*-isopropylideneinosine (**5**) in good yields. The pharmacological activities of cIDPRE and the 8-substituted derivatives (**2a**−**e**) were analyzed in intact and permeabilized human Jurkat T-lymphocytes. The results indicate that cIDPRE permeates the plasma membrane, releases Ca^{2+} from an intracellular, cADPR-sensitive Ca^{2+} store, and subsequently initiates Ca^{2+} release-activated Ca^{2+} entry. The Ca^{2+}-releasing activity of cIDPRE was confirmed directly in permeabilized cells. Using time-resolved confocal Ca^{2+} imaging at the single cell level, the development of global Ca^{2+} signals starting from local small Ca^{2+} signals evoked by cIDPRE was observed. 8-N_3-cIDPRE **2c** and 8-NH_2-cIDPRE **2d** were similarly effective in their agonistic activity as compared to cIDPRE **2a**, showing almost indistinguishable concentration−response curves for **2a**, **2c**, and **2d** and very similar kinetics of Ca^{2+} signaling. In contrast, the halogenated derivatives 8-Br- and 8-Cl-cIDPRE (**2b** and **2e**) did not significantly elevate $[Ca^{2+}]_i$. Therefore, cIDPRE **2a**, 8-N_3-cIDPRE **2c**, and 8-NH_2-cIDPRE **2d** are novel membrane permeant cADPR mimic and may provide important novel tools to study cADPR-mediated Ca^{2+} signaling in intact cells.

Introduction

Ryanodine receptors and D-myo-inositol 1,4,5-trisphosphate receptors represent the major pathways of Ca^{2+}-release from intracellular stores and have been shown to be involved in many physiological and pathological processes.[1−4] Cyclic ADP-ribose (cADPR, **1**) (Figure 1) is an endogenous nucleotide synthesized from NAD^+ by ADP-ribosyl cyclase in cells.[5] The structure of cADPR was identified by X-ray crystallography in 1994.[6] cADPR has been attracting much attention in recent years because of its potent calcium-mobilizing activities in many cellular systems, especially as a potential second messenger in cellular Ca^{2+} homeostasis in insulin secretion[7] and in T-lymphocyte activation.[8]

Many derivatives of cADPR have been synthesized from the corresponding NAD^+ analogues using ADP-ribosyl cyclase and served as valuable research tools in the elucidation of the mechanism of cADPR action,[9] e.g. labeling of binding proteins[10] or antagonizing Ca^{2+} mobilization by cADPR.[11] Specifically, cADPR analogues with substitutions at the 8-position of the adenine ring were found to be antagonists of cADPR.[11] 8-Amino-cADPR has been shown to be effective not only in sea urchin egg microsomes, but also in intact sea urchin eggs[12] as well as intact and permeabilized mammalian cells.[13,14] Another possibility to turn cADPR into an antagonist is by replacing the 3′-OH group by an 3′-*O*-methyl group.[15] Regarding modifications in the 2′-OH group significant differences between lower and higher eukaryotic systems were described: while 2′-phospho-cADPR was fully active in higher eukaryotic cells,[16,17] no Ca^{2+} release was observed in the sea urchin egg system.[18]

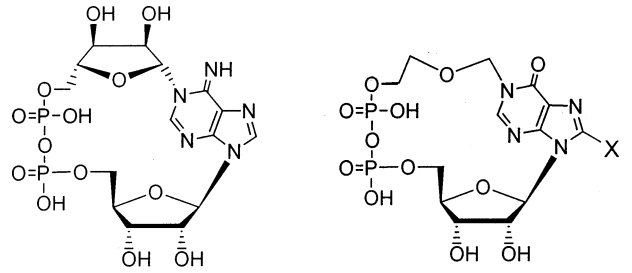

Figure 1. The structures of cADPR and 8-substituted cIDPRE.

The first nonhydrolyzable mimic of cADPR is cyclic aristeromycin-diphospho-ribose which retained a similar calcium release profile to that of cADPR.[19] Some structural modifications on adenine moiety of cADPR make the mimic more potent than its parent, e.g. 3-deaza-

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cADPR is 70-fold more potent than cADPR.[20] Additional structural modification of cADPR includes the pyrophosphate moiety, which has been replaced by triphosphate[21] and the caged compound 2-(nitrophenyl-1)ethyl pyrophosphate-cADPR;[22] unexpectedly, cyclic adenosine triphosphate ribose was more potent in inducing Ca^{2+} release as compared to cADPR. The N^1-pentyl cyclic inosine diphosphate ribose and N^1-carbocyclic, N^9-butyl analogue of cADPR were also reported.[23,24]

Shuto and co-workers first described the chemical synthesis of cyclic IDP-carbocyclic-ribose (cIDP-carboribose), in which the 4″-oxo in furanose was substituted by a methylene group[25] and reported that the corresponding stable mimic of cADPR, cADPcR, caused a significant release of Ca^{2+} in sea urchin eggs;[26] however, in mammalian cells cADPcR acted much weaker as compared to the sea urchin system.[27] Recently, a series of 8-substituted analogues of cADPcR were synthesized. In the sea urchin egg system, it was found that cADPcR was 3−4 times more potent than cADPR while the 8-substituted analogues were less efficacious.[28]

A series of N^1-glycosyl-substituted cyclic IDP-ribose derivatives were reported in our laboratory.[29,30] It was found that mimics with different configurations at the N^1-glycosyl moiety of cyclic IDP-ribose retained the cADPR activities to induce Ca^{2+} release. More interestingly, the N^1-ethoxymethyl-8-chloro-cyclic IDP-ribose analogue exhibited a strong potency to induce Ca^{2+} release in both rat brain microsomes and intact HeLa cells. This is the first mimic of cADPR with complete replacement of the terminal ribose with an ether strand, and the analogue not only remained active, but also appears to be cell permeant. Therefore, the N^1-ribosyl moiety and the 6-amino group in cADPR are not critical structural factors for retaining the biological activity. However, the relationship between the structure and agonist/antagonist behavior in cADPR and its mimics is not well understood. In contrast to N^1-ethoxymethyl-8-chloro-cyclic IDP-ribose[30] all 8-substituted cADPR analogues prepared so far were antagonists of cADPR-mediated Ca^{2+} release. However, in the case of cADPcR, 8-azido- and 8-amino-cADPcR were full agonists, although approximately 80 and 2 times less potent than cADPR, respectively.[26]

To further investigate the Ca^{2+} release activity of cyclic IDP-ribose derivatives in which the terminal ribose is replaced by an ether strand, we report here the syntheses of N^1-[(5″-*O*-phosphorylethoxy)methyl]-5′-*O*-phosphorylinosine 5′,5″-cyclicpyrophosphate (**2a;** abbreviated cIDPRE) (Figure 1) and some 8-substituted derivatives (**2a**−**e**) and their pharmacological characterizations in Jurkat T cells.

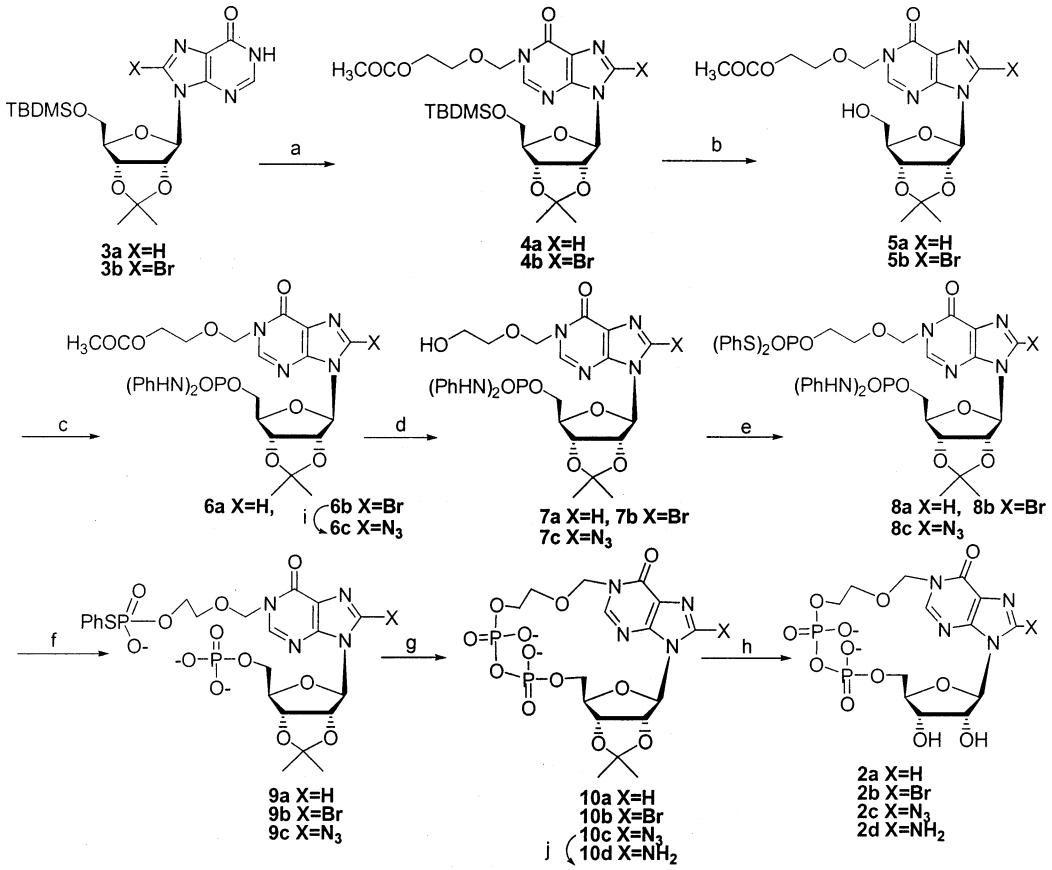
In this paper, the structural positions of cIDPRE and its derivatives are numbered as follows: a single prime numbering scheme is used for the position of the N^9-ribosyl moiety and a double prime numbering scheme is used for the position of the N^1-substitution (Figure 1).

Results and Discussion

Chemistry. The syntheses of 8-substituted cIDPRE **2a**-**2e** are summarized in Scheme 1. An N^1 substitution was carried out regioselectively on the protected inosine **3a** with 2-chloromethoxyethyl acetate in the presence of excess DBU to afford **4a** in 69% yield. The O^6-substituted side product was obtained in 19% yield. The structure of the N^1-substituted inosine **4a** was confirmed by UV, ^{1}H NMR, and ^{13}C NMR. Compared to the UV spectrum of the known compound N^1-methylinosine, compound **4a** showed a similar spectrum at 245 nm (λ_{max}) and a shoulder around 270 nm, whereas the UV spectrum of the O^6-substituted side product was different. The ^{1}H NMR spectrum of **4a** showed two doublets at 5.51 and 5.55 ppm ($H_{1''a}$, $H_{1''b}$, $J_{H1''a,\ H1''b}$ = 10.5 Hz), which coupled with C^2 in the HMBC spectrum. However, only a single peak was observed at 5.86 ppm in ^{1}H NMR spectrum of the O^6-substituted product, and no coupling peak was found in the HMBC spectrum. The above spectra data support strongly that the structure of compound **4a** is the N^1-substituted inosine derivative. After deprotection of the 5′-*tert*-butyldimethylsilyl (TBDMS) group in compound **4a**, the resulting 5′-hydroxyl of intermediate **5a** was reacted with $(PhNH)_2POCl$ to give phosphoroamide **6a** in 91% yield. ^{31}P NMR (3.06 ppm, s) indicated the signal of phosphoroamide group in compound **6a**. Deacetylation of **6a** with catalytic amount CH_3ONa gave the monohydroxy compound **7a** in 95% yield. Compound **7a** was phosphorylated with cyclohexylammonium *S,S*-diphenylphosphorodithioate (PSS) in the presence of triisopropylbenzenesulfonyl chloride (TPSCl) and tetrazole in pyridine to give **8a** in 79% yield. The presence of two different phosphate groups in the molecule was supported by ^{31}P NMR (3.21 ppm, s; 49.39 ppm, s).

The intramolecular cyclization of **9a** was completed by using Matsuda's strategy.[25,26] The selective removal of diphenylamino group in compound **8a** was carried out with isoamyl nitrite in a mixed solvent of pyridine−AcOH−Ac_2O and H_3PO_2 to give **9a,** the precursor for the intramolecular condensation reaction, in 73% yield as a triethylammonium salt. Matsuda's group suggested that introducing a bulky group into the 8-position of purine nucleosides could restrict the conformation to syn-form in which the two phosphate moieties are near each other and facilitate intramolecular condensation for the synthesis of cADPR.[25,26] Recently, they reported that in the synthesis of 8-unsubstituted cADPcR, the cyclization was performed by I_2/3 Å MS (3 Å molecular sieve) or $AgNO_3$/3 Å MS in high yield.[25,26] In the present case, the intramolecular cyclization was performed in the presence of excess I_2 and 3 Å MS in pyridine by adding a solution of compound **9a** slowly over 20 h using a syringe pump. The cyclic product **10a** was purified by HPLC as its triethylammonium salt in 71% yield. Compared to the yields of the cyclization in the syntheses of 8-Br-cIDPRE **10b** (87%) and 8-N_3-cIDPRE **10c** (87%), a more flexible N^1-ether strand in compound **9a** resulted in a larger spatial distance of the two phosphate groups resulting in a lower yield of cyclization. The cyclic structure of **10a** was confirmed by the data of HR-FABMS (523.0645, $[M - 1]^-$) and ^{31}P NMR (−9.69 and −10.61 ppm of pyrophosphate group). Finally, removal of the isopropylidene group of **10a** was carried out with aqueous HCOOH at room temperature for 8 h to furnish cIDPRE **2a**. cIDPRE **2a** was purified by two subsequent HPLC steps and characterized by ^{1}H NMR, ^{31}P NMR (−9.69 ppm, d, J = 13.4 Hz; −10.38 ppm, d, J = 13.4 Hz) and HR-FAB MS (483.0324, $[M - 1]^-$).

Scheme 1[a]



[a] Reagents and conditions: (a) DBU, $ClCH_2OCH_2CH_2OAc$, CH_2Cl_2, rt; (b) TBAF, THF, rt; (c) $(PhNH)_2POCl$, tetrazole, Py, rt; (d) CH_3ONa, CH_3OH, rt; (e) PSS, TPSCl, tetrazole, Py, rt; (f) i. isoamyl nitrite, Py:AcOH:Ac_2O (2:1:1), ii. H_3PO_2, Et_3N, Py; (g) I_2, 3 Å MS, Py, rt; (h) 60% HCOOH, rt; (i) NaN_3, DMSO, 80 °C; (j) H_2, 10% Pd/C, C_2H_5OH, rt.

To synthesize the 8-substituted cIDPRE, protected 8-bromoinosine **3b** was used as a starting material and the known procedure was followed to prepare the precursor **7b** [29] (Scheme 1). It was reported from our laboratory that the excess of TPSCl in the phosphorylation caused the substitution of bromo group at the 8-position of **7b** by chloro group.[29] Therefore, **7b** was phosphorylated with PSS, TPSCl and tetrazole in pyridine with the molecular ratio of **7b**:TPSCl to 1: 2 to afford **8b** in 87% yield. The selective deprotection of **8b** and intramolecular cyclization were carried out under a similar condition as above to give the **10b** in 88% yield. After deprotection, compound **2b** was obtained in 95% yield. 8-Bromo group of **6b** was substituted by azido group in the presence of NaN_3 in DMSO to give **6c** in 90% yield. Using the same strategy for the synthesis of cIDPRE **2a, 10c** was obtained in 89% yield and **2c** was in 93% yield from **10c**. **10c** was hydrogenated under the catalyst of 10% Pd/C to give **10d** in 99% yield. After removing the protecting group, **2d** was obtained in 90% yield.

Pharmacology. The pharmacological activities of cIDPRE **2a** and 8-Br- **2b**, 8-N_3- **2c**, 8-NH_2- **2d**, 8-Cl-cIDPRE **2e**[30] were analyzed in intact and permeabilized human Jurkat T-lymphocytes. Addition of cIDPRE **2a** to intact cells in the presence of extracellular Ca^{2+} activated a concentration-dependent biphasic pattern of Ca^{2+} mobilization, starting with a small rise that was then followed by a much larger increase (Figure 2A,B). The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) remained elevated for at least 15 min. Since even at 1 mM cIDPRE **2a** saturation of the response was not achieved (Figure 2B), no EC_{50} can be given. Using a Ca^{2+} free/Ca^{2+} reintroduction protocol, cIDPRE **2a** activated a transient Ca^{2+} release from intracellular Ca^{2+} stores (Figure 2C). Readdition of Ca^{2+} after the signal had returned to the baseline rapidly activated Ca^{2+} entry from the extracellular space resulting in a sustained elevation of $[Ca^{2+}]_i$ (Figure 2C). These results indicate that cIDPRE **2a** permeates the plasma membrane, releases Ca^{2+} from an intracellular, cADPR-sensitive Ca^{2+} store, and subsequently initiates Ca^{2+} release-activated Ca^{2+} entry. The Ca^{2+} releasing activity of cIDPRE **2a** was confirmed directly in permeabilized cells (Figure 2D, lower panel); quantitative analysis resulted in a lower efficacy of cIDPRE **2a** as compared to cADPR **1** (Figure 2D, upper panel).

One of the hallmarks of Ca^{2+} signaling is the development of global Ca^{2+} signals starting from local subcellular pacemaker signals. Using confocal Ca^{2+} imaging at the single cell level, upon extracellular addition of cIDPRE **2a**, we demonstrate the induction of localized small Ca^{2+} signals close to the cell border (Figure 2E; image at 138 s; arrow). With time isolated neighboring Ca^{2+} signals merge into larger signals reflecting recruitment of additional Ca^{2+} release microdomains (Figure 2E; image at 147 and 148 s). Finally, individual signals are almost indistinguishable from each other: the signal becomes global (Figure 2E; image at 153 s).

Modification of cIDPRE at C8 of inosine had strikingly differential effects. 8-N_3-cIDPRE **2c** (Figure 3B) and 8-NH_2-cIDPRE **2d** (Figure 3C) were similarly effective

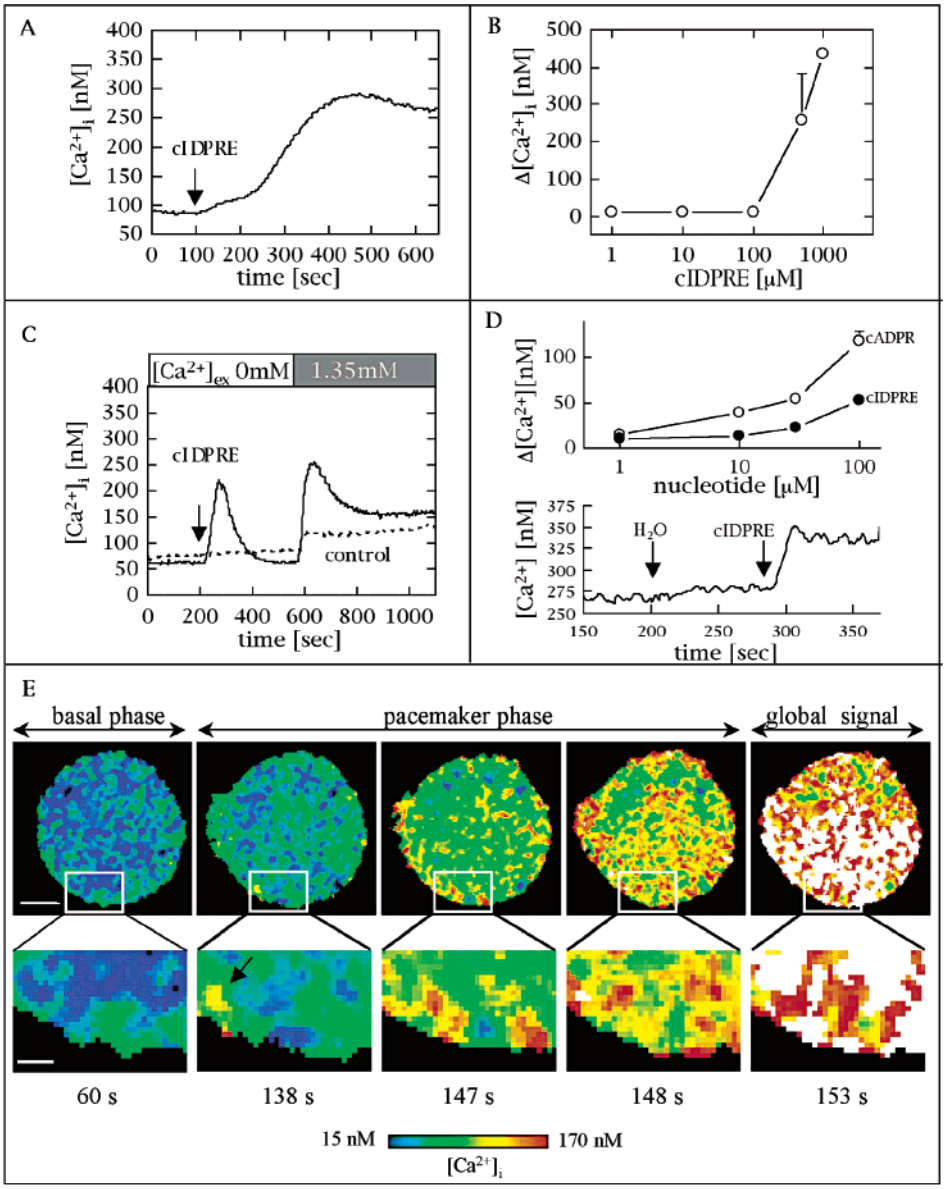


Figure 2. Effect of the cADPR mimic cIDPRE on Ca^{2+} signaling in T cells. Intact Jurkat T cells were loaded with Fura2/AM (A−C, E), and $[Ca^{2+}]_i$ was determined fluorimetrically in cell suspension (A−C) or by confocal Ca^{2+} imaging on the single cell level (E) as described in the Experiment Section. A: cIDPRE was added as indicated (final concentration 0.5 mM); a representative tracing out of five experiments is displayed. B: Concentration−response data are given as mean ± SEM (n = 3 to 5; error bars are partially not visible because the values are smaller than the symbol size). C: Cells were kept in a nominal Ca^{2+} free buffer in the first part of the experiment; cIDPRE was added as indicated. Then, $CaCl_2$ was readded. The dashed line shows a control experiment where buffer was added instead of cIDPRE. D: Cells were permeabilized as described in the experiment section. Vehicle (H_2O) and cIDPRE (100 μM) were added as indicated (lower panel). Upper panel: data are mean ± SEM (n ≥ 3; error bars are partially not visible because the values are smaller than the symbol size). E: Characteristic confocal ratiometric pseudocolor images of a single Jurkat cell and magnifications of a defined subcellular region are shown. Basal phase (before addition of 0.5 mM cIDPRE [final concentration]), pacemaker phase and global phase are indicated. White bar corresponds to 3 μm in the upper panel and to 1 μm in the lower panel. In total 15 cells were analyzed.

in their agonistic activity as compared to cIDPRE **2a** (Figure 3A−C); note the quantitatively almost indistinguishable concentration−response curves for **2a**, **2c** and **2d** (Figure 3D) and the very similar kinetics of Ca^{2+} signaling (Figure 3A-C). In contrast, the halogenated derivatives 8-Br- and 8-Cl-cIDPRE (compounds **2b** and **2e**) did not significantly elevate $[Ca^{2+}]_i$ (Figure 3D). These data indicate that the amino or the azido group at the 8-position of the inosine ring much better complies with the structural requirements for an agonist as compared to a halogen group.

In addition to the previously described N^1-glycosyl-substituted cyclic IDP-ribose derivatives[29,30] and 8-Br-cIDPR,[31] the compounds described in the present study, cIDPRE **2a** and 8-NH_2- and N_3-cIDPRE (compounds **2d** and **2c**), are novel and important tools similarly suitable for studying the structure−activity relationship of cADPR and to be used as a membrane permeant cADPR mimics in intact cells. The cADPR/Ca^{2+} signaling pathway has been demonstrated in many different cell types and tissues (see Table 1 in ref 4); however, in most of the cases either cADPR had to be delivered into intact cells by microinjection[32] or infusion via a patch-clamp pipet,[33] or broken cell preparations such as permeabilized cells[13] or cell homogenates[34] were used. Clearly, the advantage of a membrane-permeant mimic is that

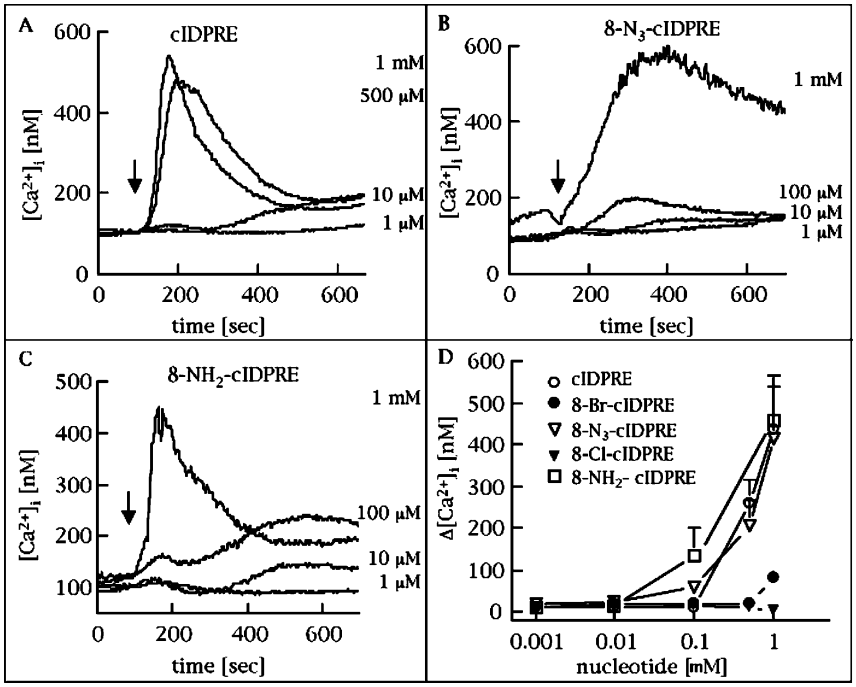


Figure 3. Effect of the cIDPRE and 8-substituted cIDPRE analogues on Ca^{2+} signaling in T cells. Intact Jurkat T cells were loaded with Fura2/AM, and $[Ca^{2+}]_i$ was determined fluorimetrically in cell suspension as described in the Experiment Section. Different concentrations of cIDPRE (A), 8-N_3-cIDPRE (B) and 8-NH_2-cIDPRE (C) were added (arrow) as indicated. Mean values ± SEM (n = 3 to 5) are displayed as concentration−response curve in D.

the cells remain intact and mechanically untouched. This is especially important in cell types where mechanical influences would activate (or inhibit) Ca^{2+} signaling on their own. Thus, time-resolved subcellular Ca^{2+} imaging is technically less difficult using a membrane permeant cADPR mimic simply added to the extracellular medium as compared to microinjection of the natural compound cADPR. A further advantage is that the involvement of the cADPR/Ca^{2+} signaling system can be studied in a larger number of cells, e.g. in cell suspension, thus providing a more accurate mean of the cellular response.

Comparison of the different cIDPRE derivatives tested here revealed a remarkable structure−activity relationship: while any functional group larger than the natural H-atom at C8 in cADPR turned the molecule into a cADPR antagonist, e.g. 8-NH_2-cADPR,[11,13] 8-Br-cADPR,[11,13] 8-OCH_3-cADPR,[8] 8-CH_3-cADPR,[17] in the case of cyclic IDP-ribose mimics, different functional groups at the 8-position resulted in completely different biological activities. In our case, the electronegative action of halogen atoms may either directly prevent binding at the cADPR binding pocket or may influence the overall conformation of the whole molecule in a way that it does not resemble cADPR any more. The latter may be due to a greater flexibility of the whole molecule introduced by replacement of the terminal ribose by the ether strand. In contrast, the 8-N_3- and 8-NH_2-analogues (compounds **2c** and **2d**) either still fit into the binding site or the functional groups support a conformation much more like cADPR. Specifically, the amino ligand at the 8-position of the inosine ring of cIDPRE may contribute as a proton donor for the formation of a hydrogen bond with the neighboring amino acid residue of the receptor. The longer lasting activity of 8-N_3-cIDPRE **2c** as compared to cIDPRE or 8-NH_2-cIDPRE (Figure 3B vs Figures 3A and 3C) may, in addition, be due to UV-induced formation of covalent bonds between this ligand and its receptor protein.

Taken together, chemical synthesis of cIDPRE and its analogues resulted in important novel tools to study cADPR-mediated Ca^{2+} signaling in intact cells and new and unexpected insights into the structure−activity relationship of cADPR.

Experiment Section

Chemistry. UV spectra were recorded on a Varian DMS200 UV−visible spectrophotometer. Mass spectra were obtained on either VG-ZAB-HS or Bruker APEX. High-resolution FAB (fast atom bombardment) mass and HR EIMS (electrospray ionization) were performed with Bruker BIFLEX III. ^{1}H NMR and ^{13}C NMR were recorded with a JEOL AL300 or a Varian VXR-500 spectrometer using DMSO-d_6 or D_2O as a solvent. Chemical shifts are reported in parts per million downfield from TMS (^{1}H and ^{13}C). ^{31}P NMR spectra were recorded at room temperature by use of Bruker Avance 300 spectrometer (121.42 MHz); Orthophosphoric acid (85%) was used as an external standard. Compounds (**2a**−**d**, **9a**−**c** and **10a**−**d**) were purified on Alltech preparative C18 reversed phase column (2.2 × 25 cm) with Gilson HPLC by two different buffer systems: MeCN/TEAA (pH 7.0) or MeCN/TEAB (pH 7.5).

***N*^{1}-[(5″-Acetoxyethoxy)methyl]-5′-*O*-TBDMS-2′,3′-*O*-isopropylideneinosine (4a).** To the solution of **3a** (422 mg, 1 mmol) and DBU (1.4 mL, 10 mmol) in CH_2Cl_2 (10 mL) was added $ClCH_2OCH_2CH_2OAc$ (0.7 mL, 5 mmol) at 0°C. After being stirred for 30 min, the solvent was evaporated in vacuo and the residue was purified by silica gel column chromatography (hexane−acetone) to give compound **4a** (371 mg, 69%). ^{1}H NMR (300 MHz, DMSO) δ 0.00 (s, 6H, $(CH_3)_2Si$), 0.82 (s, 9H, $(CH_3)_3C$-), 1.31, 1.52 (each s, each 3H, $(CH_3)_2C$), 1.90 (s, 3H, AcO), 3.77−3.87 (m, 4H, OCH_2, 2 × $H_{5'}$), 4.09−4.13 (m, 2H, CH_2OAc), 4.28−4.29 (m, 1H, $H_{4'}$), 4.99 (dd, 1H, $J_{H3',H4'}$ = 3.0, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.28 (dd, 2H, $J_{H1',H2'}$ = 2.5, $J_{H2',H3'}$ = 6.0 Hz, $H_{2'}$), 5.51 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''a}$), 5.55 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''b}$), 6.14 (d, 1H, $J_{H2',H1'}$ = 2.5 Hz, $H_{1'}$), 8.15, 8.36 (each s, each 1H, H_2, H_8); ^{13}C NMR (75 MHz, DMSO) δ 170.21, 155.98, 148.90, 146.95, 139.38, 123.89, 113.12, 89.75, 86.85, 83.94, 81.01, 74.68, 67.00, 63.12, 62.89,

26.92, 25.69, 25.14, 20.54, 17.92, −5.57; UV (MeOH) λ_{max} 207 nm, 245 nm, sh 270 nm. Anal. ($C_{24}H_{37}N_4O_8Si$) C, H, N.

***N*¹-[(5″-Acetoxyethoxy)methyl]-2′,3′-*O*-isopropylideneinosine (5a).** A solution of **4a** (360 mg, 0.67 mmol), and TBAF (1 M in THF, 1.3 mL, 1.3 mmol) in THF (5 mL) was stirred for 2 h at room temperature under neutral conditions. The resulting mixture was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography (hexane-acetone) to give **5a** (256 mg, 90%): ^{1}H NMR (300 MHz, DMSO) δ 1.32, 1.54 (each s, each 3H, $(CH_3)_2C$-), 1.94 (s, 3H, AcO), 3.53−3.56 (m, 2H, 2 × $H_{5'}$), 3.74−3.77 (m, 2H, OCH_2), 4.08−4.11 (m, 2H, CH_2OAc,), 4.23−4.24 (m, 1H, $H_{4'}$), 4.94 (dd, 1H, $J_{H3',H4'}$ = 2.1 Hz, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.12 (t, 1H, $J_{OH,H5'}$ = 5.1, OH), 5.27 (dd, 1H, $J_{H1',H2'}$ = 2.1, $J_{H3',H2'}$ = 6.0 Hz, $H_{2'}$), 5.46 (s, 2H, $H_{1''}$), 6.11 (d, 1H, $J_{H1',H2'}$ = 2.1 Hz, $H_{1'}$), 8.36, 8.51; (each s, each 1H, H_2, H_8); ^{13}C NMR (75 MHz, DMSO) δ 170.27, 156.00, 149.01, 147.11, 139.46, 123.72, 113.11, 89.62, 86.77, 83.86, 81.25, 74.73, 67.05, 62.94, 61.41, 26.99, 25.13, 20.55; Anal. ($C_{18}H_{29}N_4O_8$) C, H, N.

***N*¹-[(5″-Acetoxyethoxy)methyl]-5′-*O*-dianilinophosphoryl-2′,3′-*O*-isopropylideneinosine (6a).** To a solution of **5a** (350 mg, 0.825 mmol) and tetrazole (231 mg, 3.302 mmol) in dry pyridine (5 mL) was added $(PhNH)_2POCl$ (880 mg, 3.302 mmol) at room temperature, and the mixture was stirred for 48 h. The solvent was evaporated to dryness, and the residue was purified by silica gel column chromatography (CH_2Cl_2−MeOH) to afford **6a** (487 mg, 91%) as a white foam. ^{1}H NMR (300 MHz, DMSO) δ 1.29, 1.52 (each s, each 3H, CH_3 × 2), 1.94 (s, 3H, AcO), 3.73−3.77 (m, 2H, 2 × $H_{5'}$), 4.07−4.19 (m, 4H, CH_2OAc, OCH_2), 4.43 (m, 1H, $H_{4'}$), 4.99 (dd, 1H, $J_{H3',H4'}$ = 3.0, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.25 (dd, 1H, $J_{H2',H1'}$ = 2.4, $J_{H2',H3'}$ = 6.0 Hz, $H_{2'}$), 5.40 (d, 1H, $J_{H1''a,H1''b}$ = 10.4 Hz, $H_{1''a}$), 5.46 (d, 1H, $J_{H1''a,H1''b}$ = 10.4 Hz, $H_{1''b}$) 6.15 (d, 1H, $J_{H1',H2'}$ = 2.4 Hz, $H_{1'}$), 6.75−7.16 (m, 10H, Ar H), 8.07, 8.11 (each d, each1H, $J_{NHa,NHb}$ = 9.9 Hz, NH × 2), 8.28, 8.39 (each s, each 1H, H_8, H_2). ^{13}C NMR (75 MHz, DMSO) δ 170.32, 156.00,148.95, 146.98, 141.04, 140.98, 139.55, 128.81, 128.76, 123.96, 120.52, 117.35, 117.25, 113.67, 113.61, 89.35, 84.47, 83.55, 80.93, 74.82, 67.13, 64.44, 62.96, 26.93, 25.13, 20.58; ^{31}P NMR (DMSO, 81 MHz, decoupled with ^{1}H) δ 3.06 (s). Anal. ($C_{30}H_{36}N_6O_9P$·2CH_3OH) C, H, N.

***N*¹-(5″-Hydroxylethoxymethyl)-5′-*O*-(dianilinophosphoryl)-2′,3′-*O*-isopropylideneinosine (7a).** Compound **6a** (460 mg, 0.703 mmol) was dissolved in 10 mL of methanol. To the solution was added CH_3ONa (8 mg, 0.14 mmol) and stirred for 2 h. Dowex-50W acid cation-exchange resin was added to adjust pH to 7 and then filtered, and solvent was removed under reduced pressure to give **7a** (409 mg, 95%): ^{1}H NMR (300 MHz, DMSO) δ 1.28,1.51 (each s, each 3H, CH_3 × 2), 3.46−3.49 (m, 2H, 2 × $H_{5'}$), 3.54−3.57 (m, 2H, OCH_2-), 4.07−4.22 (m, 2H, CH_2OH), 4.43 (m, 1H, $H_{4'}$), 4.99 (dd, 1H, $J_{H3',H4'}$ = 3.0, $J_{H3',H2'}$ = 6.0 Hz, $H_{3'}$), 5.25 (dd, 1H, $J_{H1',H2'}$ = 2.5, $J_{H2',H3'}$ = 6.0 Hz, $H_{2'}$), 5.39 (d, 1H, $J_{H1''a,H1''b}$ = 10.4 Hz, $H_{1''a}$), 5.46 (d, 1H, $J_{H1''a,H1''b}$ = 10.4, $H_{1''b}$), 6.15 (d, 1H, $J_{H1',H2'}$ = 2.5 Hz, $H_{1'}$), 6.75−7.15 (m, 10H, Ar H), 8.06, 8.09 (each d, each1H, $J_{NHa,NHb}$ = 6.3 Hz, NH × 2), 8.27, 8.37 (each s, each 1H, H_8, H_2); ^{13}C NMR (75 MHz, DMSO) δ 155.95, 148.89, 146.95, 141.00, 140.96, 139.50, 128.81, 128.75, 123.95, 120.52, 117.34, 117.25, 113.58, 89.34, 84.48, 83.54, 80.91, 75.00, 70.93, 64.51, 60.05, 26.93, 25.13; ^{31}P NMR (DMSO, 81 MHz, decoupled with ^{1}H) δ 3.17. Anal. ($C_{28}H_{33}N_6O_8P$) C, H, N.

***N*¹-[[[Bis-5″-(phenylthio)phosphoryl]oxyethoxy]methyl]-5′-*O*-(dianilinophosphoryl)-2′,3′-*O*-isopropylideneinosine (8a)** Compound **7a** (400 mg, 0.653 mmol) was dissolved in dry pyridine (7 mL) and then added TPSCl (395 mg, 1.31 mmol), PSS (742 mg, 1.96 mmol) and tetrazole (137 mg, 1.96 mmol). The resulting solution was stirred for 12 h at room temperature. After removed of the solvent, the residue was purified by silica gel column chromatography (hexane-acetone) to give compound **8a** (452 mg, 79%). ^{1}H NMR (300 MHz, DMSO) δ 1.31,1.56 (each s, each 3H, CH_3 × 2), 3.85 (m, 2H, 2 × $H_{5'}$), 4.15−4.23 (m, 2H, OCH_2-), 4.35 (m, 2H, CH_2OP), 4.47 (m, 1H, $H_{4'}$), 5.03 (dd, 1H, $J_{H3',H4'}$ = 3.0, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.27 (dd, 1H, $J_{H1',H2'}$ = 2.4, $J_{H2',H3'}$ = 6.0 Hz, $H_{2'}$), 5.38 (d, 1H, $J_{H1''a,H1''b}$ = 10.2 Hz, $H_{1''a}$), 5.48 (d, 1H, $J_{H1''a,H1''b}$ = 10.2 Hz, $H_{1''b}$), 6.19 (d, 1H, $J_{H1',H2'}$ = 2.4 Hz, $H_{1'}$), 6.80−7.57 (m, 20H, Ar H), 8.13, 8.16 (each d, each1H, $J_{NHa,NHb}$ = 6.3 Hz, NH × 2), 8.33, 8.43 (each s, each 1H, H_8,H_2); ^{31}P NMR (DMSO, 81 MHz, decoupled with ^{1}H) 3.21 ppm (s); 49.39 ppm (s). ESI-TOF+-MS: *m/z* = 877.2373 [(M + 1)$^+$]; Anal. ($C_{40}H_{42}N_6O_9P_2S_2$·1/2hexane) C, H, N.

***N*¹-[[[5″-(Phenylthio)phosphoryl]oxyethoxy]methyl]-5′-*O*-phosphoryl-2′,3′-*O*- isopropylideneinosine (9a).** A solution of **8a** (120 mg, 0.137 mmol) and isoamyl nitrite (277 μL, 2.05 mmol) in pyridine−AcOH−Ac_2O (2:1:1, v/v, 4 mL) was stirred at room temperature for 8 h. After the reaction mixture was evaporated (at <30 °C), the residue was dissolved with H_3PO_2 (140 μL, 2.74 mmol) and Et_3N (190 μL, 1.37 mmol) in pyridine (3 mL), and the resulting solution was stirred for 11 h at room temperature. After the solvent was evaporated to dryness under reduced pressure, the residue was partitioned between $CHCl_3$ and H_2O. The aqueous layer was coevaporated (<30 °C) with pyridine (5 mL), and the residue was dissolved in TEAA (0.1 M, pH 7.0, 5 mL). The solution was purified by a C18 reverse phase column (2.2 × 25 cm) using a linear gradient of 0−40% CH_3CN in TEAA buffer (0.1 M, pH 7.0) within 30 min to give **9a** as a triethylammonium salt. The compound was purified again using same column eluting with MeCN/TEAB buffer (pH 7.5) (84 mg, 73%). ^{1}H NMR (500 MHz, D_2O) δ 1.45, 1.67 (each s, each 3H, 2 × CH_3), 3.87−3.88 (m, 2H, $H_{5'}$), 4.05−4.10 (m, 2H, CH_2O), 4.11−4.13 (m, 3H, $H_{4'}$, CH_2OP), 5.15 (dd, 1H, $J_{H3',H4'}$ = 2.0, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.31 (dd, 1H, $J_{H2',H1'}$ = 3.0, $J_{H3',H2'}$ = 6.0 Hz, $H_{2'}$), 5.50 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''a}$), 5.56 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''b}$), 6.21 (d, 1H, $J_{H1',H2'}$ = 3.0 Hz, $H_{1'}$), 7.18−7.43 (m, 5H, Ar H), 8.38, 8.43 (each s, each1H, H_2, H_8); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ 2.06(s), 17.79(s). HRMS (TOF, negative) for $C_{22}H_{28}N_4O_{12}P_2S$: Calcd, 633.0827 [(M − 1)$^-$]; found, 633.0818.

***N*¹-[(5″-*O*-Phosphorylethoxy)methyl]-2′,3′-*O*-isopropylidene 5′-*O*-phosphorylinosine 5′,5″-cyclicpyrophosphate (10a).** A solution of **9a** (30 mg, 36 μmol) in pyridine (5 mL) was added slowly over 20 h, using a syringe pump, to a mixture of I_2 (190 mg) and MS 3 Å (1.9 g), in pyridine (50 mL) at room temperature in the dark. The MS 3 Å was filtered off with Celite and washed with H_2O. The combined filtrate was evaporated, and the residue was partitioned between $CHCl_3$ and H_2O. The aqueous layer was evaporated, and the residue was dissolved in 0.1 M TEAA buffer (1.0 mL), which was applied to C18 reversed phase column (2.2 × 25 cm). The column was developed using a linear gradient of 0−80% CH_3CN in TEAA buffer (0.1 M, pH 7.0) within 30 min to give **10a** as a triethylammonium salt. The compound was purified again using same column eluting with MeCN/TEAB buffer (pH 7.5) (18.5 mg, 71%). ^{1}H NMR (500 MHz, D_2O) δ 1.47, 1.63 (each s, each 3H, 2 × CH_3), 3.72−3.77 (m, 1H, $H_{5a'}$), 3.86−3.89 (m, 2H, CH_2O), 3.92−3.97 (m, 3H, $H_{5'b}$, CH_2OP), 4.60 (m, 1H, $H_{4'}$), 5.35 (dd, 1H, $J_{H3',H4'}$ = 2.0, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.49 (d, 1H, $J_{H1''a,H1''b}$ = 11.5, $H_{1''a}$), 5.88 (d, 1H, $J_{H1''a,H1''b}$ = 11.5, $H_{1''b}$), 5.92 (d, 1H, $J_{H2',H3'}$ = 6.0, $H_{2'}$), 6.33 (s,1H, $H_{1'}$), 8.22, 8.49 (each s, each 1H, H_2, H_8); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ −9.69, −10.61. HRMS (FAB, negative) for $C_{16}H_{22}N_4O_{12}P_2$ Calcd, 523.0637 [(M − 1)$^-$]; found, 523.0645.

***N*¹-[(5″-*O*-Phosphorylethoxy)methyl]-5′-*O*-phosphorylinosine 5′,5″-Cyclicpyrophosphate (cIDPRE 2a).** The solution of **10a** (18.5 mg, 25.5 μmol) in 60% HCOOH (5 mL) was stirred for 8 h and then evaporated under reduced pressure. The purification of the residue was performed at the same procedure as compound **10a** by twice HPLC on C18 reverse column eluting with MeCN/TEAA (pH 7.0) and MeCN/TEAB (pH 7.5) buffer, respectively, to give the target molecule **2a** (16 mg. 91%): ^{1}H NMR (500 MHz, D_2O) δ 3.75−3.86 (m, 3H, $H_{5a'}$, CH_2O-), 3.88−3.96 (m, 2H, CH_2OP), 4.04−4.06 (m, 1H, $H_{5'b}$), 4.19−4.23 (m, 1H, $H_{4'}$), 4.30−4.32 (1H, m, $H_{3'}$), 5.43 (t, 1H, $J_{H1',H2'}$ = 4.0 Hz, $J_{H3',H2'}$ = 4.0 Hz, $H_{2'}$), 5.48 (d, 1H, $J_{H1''a,H1''b}$ = 11.5 Hz, $H_{1''a}$), 5.80 (d, 1H, $J_{H1''a,H1''b}$ = 11.5 Hz, $H_{1''b}$), 6.05 (d, 1H, $J_{H1',H2'}$ = 4.0 Hz, $H_{1'}$), 8.19, 8.50 (each s, each 1H, H_2, H_8); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ −9.69 (d,

$J_{P,P}$ = 13.4 Hz), −10.38 (d, $J_{P,P}$ = 13.4 Hz); HRMS (FAB, negative) for $C_{13}H_{18}N_4O_{12}P_2$ Calcd, 483.0324 [(M − 1)$^-$]; found, 483.0324.

***N*^{1}-[[[Bis-5″-(phenylthio)phosphoryl]oxyethoxy]methyl]-5′-*O*-(dianilinophosphoryl)-2′,3′-*O*-isopropylidene-8-bromoinosine (8b).** Compound **6b** (200 mg, 0.27 mmol)[25] was dissolved in 10 mL of methanol. To the solution was added CH_3ONa (4 mg, 0.07 mmol) and stirred at room temperature for 2h. Dowex-50W acid cation-exchange resin was added to adjust the solution to pH 7, and then filtered and removed of the solvent under reduce pressure to give **7b.** Compound **7b** was dissolved in dry pyridine (15 mL) and then added TPSCl (164 mg, 0.54 mmol), PSS (308 mg, 0.81 mmol) and tetrazole (57 mg, 0.81 mmol). The resulting solution was stirred for 12 h at room temperature. After removed of the solvent, the residue was purified by silica gel column chromatography (hexane-acetone) to give compound **8b** (224 mg, 87%). ^{1}H NMR (300 MHz, DMSO) δ 1.51,1.29 (each s, each 3H, CH_3 × 2), 3.80 (m, 2H, 2 × $H_{5'}$), 4.07−4.37 (m, 5H, OCH_2-, CH_2OP, $H_{4'}$), 5.06 (dd, 1H, $J_{H3',H4'}$ = 3.0, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.30 (d, 1H, $J_{H1''a,H1''b}$ = 10.2 Hz, $H_{1''a}$), 5.45 (dd, 1H, $J_{H1',H2'}$ = 2.4, $J_{H2',H3'}$ = 6.0 Hz, $H_{2'}$), 5.49 (d, 1H, $J_{H1''a,H1''b}$ = 10.2 Hz, $H_{1''b}$), 6.02 (d, 1H, $J_{H1',H2'}$ = 2.4 Hz, $H_{1'}$), 6.72−7.53 (m, 20H, Ar H), 8.01, 8.06 (each d, each1H, $J_{NHa,NHb}$ = 12.6 Hz, NH × 2), 8.30 (s, 1H, H_2); ^{31}P NMR (DMSO, 81 MHz, decoupled with ^{1}H) 2.80 ppm (s); 51.08 ppm (s). ESI-TOF+-MS: *m/z* = 956.2284 [(M + 1)$^+$]; Anal. ($C_{40}H_{41}N_6O_9BrP_2S_2$·2acetone) C, H, N.

***N*^{1}-[[[5″-(Phenylthio)phosphoryl]oxyethoxy]methyl]-5′-*O*-phosphoryl-2′,3′-*O*- isopropylidene-8-bromoinosine (9b).** A solution of **8b** (115 mg, 0.12 mmol) and isoamyl nitrite (243 μL, 1.80 mmol) in pyridine−AcOH−Ac_2O (2:1:1, v/v, 4 mL) was stirred at room temperature for 8 h. After the reaction mixture was evaporated (at <30 °C), the residue was dissolved with H_3PO_2 (123 μL, 2.4 mmol) and Et_3N (167 μL, 1.2 mmol) in pyridine (3 mL), and the resulting solution was stirred for 11 h at room temperature. After the solvent was evaporated to dryness under reduced pressure, the residue was partitioned between $CHCl_3$ and H_2O. The aqueous layer was coevaporated (<30 °C) with pyridine (5 mL), and the residue was purified with the same procedure shown in the preparation of **9a** to give **9b** as a triethylammonium salt. The compound was purified again using same column eluting with MeCN/TEAA buffer (pH 7.0) (93 mg, 85%). ^{1}H NMR (500 MHz, D_2O) δ 1.45, 1.65 (each s, each 3H, 2 × CH_3), 3.70 (m,1H, $H_{5'a}$), 3.92 (m, 3H, CH_2O, $H_{5'b}$), 4.08−4.12 (m, 3H, $H_{4'}$, CH_2OP), 5.26 (dd, 1H, $J_{H3',H4'}$ = 2.0, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.39 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''a}$), 5.51 (dd, 1H, $J_{H2',H1'}$ = 3.0, $J_{H3',H2'}$ = 6.0 Hz, $H_{2'}$), 5.67(d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''b}$), 6.25 (d, 1H, $J_{H1',H2'}$ = 3.0 Hz, $H_{1'}$), 7.18−7.52 (m, 5H, Ar H), 8.38 (s, 1H, H_2); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ 2.84(s), 17.69(s). HRMS (TOF, negative) for $C_{22}H_{27}N_4O_{12}BrP_2S$: Calcd, 712.3780-[(M − 1)$^-$]; found, 711.9582.

***N*^{1}-[(5″-*O*-Phosphorylethoxy)methyl]-2′,3′-*O*-isopropylidene-5′-*O*-phosphoryl-8-bromoinosine 5′,5″-Cyclicpyrophosphate (10b).** A solution of **9b** (25 mg, 27 μm) in pyridine (5 mL) was added slowly over 20 h, using a syringe pump, to a mixture of I_2 (144 mg) and MS 3 Å (1.44 g), in pyridine (40 mL) at room temperature in the dark. The purification of **10b** was same as the preparation of **10a** and **10b** was obtained as a triethylammonium salt. The compound was purified again using same column eluting with MeCN/TEAB buffer (pH 7.5) (19 mg, 87% yield). ^{1}H NMR (500 MHz, D_2O) δ 1.46, 1.63 (each s, each 3H, 2 × CH_3), 3.79−3.86 (m, 3H, CH_2O, $H_{5a'}$), 3.92−3.95 (m, 3H, $H_{5'b,}$ CH_2OP), 4.59 (m, 1H, $H_{4'}$), 5.25 (d, 1H, $J_{H1''a,H1''b}$ = 11.5 Hz, $H_{1''a}$) 5.49 (d, 1H, $J_{H2',H3'}$ = 4.0 Hz, $H_{3'}$), 5.98−6.04 (m, 2H, $H_{1''b}$, $H_{2'}$), 6.33 (s,1H, $H_{1'}$), 8.52, (s, 1H, H_2); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ −9.65(d, $J_{P,P}$ = 10.0 Hz), −10.35(d, $J_{P,P}$ = 10.0 Hz). HRMS (FAB, negative) for $C_{16}H_{21}BrN_4O_{12}P_2$ Calcd, 600.9815 [(M − 1)$^-$]; found, 600.9735.

***N*^{1}-[(5″-*O*-Phosphorylethoxy)methyl]-5′-*O*-phosphoryl-8- bromoinosine 5′,5″-Cyclicpyrophosphate (2b).** The solution of **10b** (10 mg, 12 μmol) in 60% HCOOH (4 mL) was stirred for 8 h, and then evaporated under reduced pressure. The purification of the residue was performed at the same procedure as compound **2a** by twice HPLC on C18 reverse column eluting with MeCN/TEAB (pH 7.5) and MeCN/TEAA (pH 7.0) buffer respectively to give the target molecule **2b** (8.5 mg. 89%). ^{1}H NMR (500 MHz, D_2O) δ 3.77−3.85 (m, 2H, CH_2O-), 3.93−4.00 (m, 2H, OCH_2OP), 4.03−4.09 (m, 1H, $H_{5a'}$), 4.30−4.32 (2H, m, $H_{5b'}$, $H_{4'}$), 4.85 (m, 1H, $H_{3'}$), 5.25 (d, 1H, $J_{H1''a,H1''b}$ = 11.0 Hz, $H_{1''a}$), 5.68(t, 1H, $J_{H1',H2'}$ = 5.0 Hz, $J_{H3',H2'}$ = 5.0 Hz, $H_{2'}$), 5.99 (d, 1H, $J_{H1''a,H1''b}$ = 11.0 Hz, $H_{1''b}$), 6.11(d, 1H, $J_{H1',H2'}$ = 5.0 Hz, $H_{1'}$), 8.51 (s, 1H, H_2); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ −9.70 (d, $J_{P,P}$ = 10.0 Hz), −10.35 (d, $J_{P,P}$ = 10.0 Hz); HRMS (TOF, negative) for $C_{13}H_{17}BrN_4O_{12}P_2$ Calcd, 560.9502 [(M − 1)$^-$]; found, 560.9449.

***N*^{1}-[[[Bis-5″-(phenylthio)phosphoryl]oxyethoxy]methyl]-5′-*O*-(dianilinophosphoryl)-2′,3′-*O*-isopropylidene-8-azidoinosine (8c).** Compound **6b** (350 mg, 0.48 mmol) and NaN_3 (100 mg, 1.52 mmol) were dissolved in 5 mL of anhydrous DMSO, the mixture was reacted at 80 °C for 12 h under argon. After the removal of DMSO, the residue was extracted with CH_2Cl_2 (3 mL x 3). The extract was purified on silica gel column (petroleum ether: acetone = 3:1) to give 300 mg of **6c** in 90% yield. Compound **6c** (200 mg, 0.29 mmol) was dissolved in 10 mL of methanol. To the solution was added CH_3ONa (4 mg, 0.07 mmol) and stirred at room temperature for 2h. Dowex-50W acid cation-exchange resin was added to adjust pH to 7, and then filtered and removed of the solvent under reduced pressure to give **7c.** Compound **7c** was dissolved in dry pyridine (10 mL) and then added TPSCl (179 mg, 0.59 mmol), PSS (354 mg, 0.93 mmol) and tetrazole (65 mg, 0.93 mmol). The resulting solution was stirred for 12 h at room temperature. After removal of the solvent, the residue was purified by silica gel column chromatography (hexane-acetone) to give compound **8c** (237 mg, 80%). ^{1}H NMR (300 MHz, DMSO) δ 1.26,1.49 (each s, each 3H, CH_3 × 2), 3.82 (m, 2H, 2 × $H_{5'}$), 4.00−4.33 (m, 5H, OCH_2-, CH_2OP, $H_{4'}$), 5.05 (dd, 1H, $J_{H3',H4'}$ = 3.0, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.32 (dd, 1H, $J_{H1',H2'}$ = 2.4, $J_{H2',H3'}$ = 6.0 Hz, $H_{2'}$), 5.38 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''a}$) 5.45 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''b}$), 5.89 (d, 1H, $J_{H1',H2'}$ = 2.4 Hz, $H_{1'}$), 6.76−7.52 (m, 20H, Ar H), 8.08 (t, $J_{NHa,NHb}$ = 9.9 Hz, NH × 2), 8.34 (s, 1H, H_2); ^{13}C NMR (75 MHz,DMSO) δ 154.75, 148.56, 146.82, 143.42, 141.07, 135.07, 135.01, 129.70, 128.74, 128.62, 125.67, 125.58, 121.50, 120.38, 117.25, 117.17, 117.08, 113.68, 87.68, 85.18, 82.71, 80.64, 74.97, 68.11, 66.97, 64.17, 26.92, 25.17. ^{31}P NMR (DMSO, 81 MHz, decoupled with ^{1}H) 3.21 ppm (s); 49.37 ppm (s). ESI-TOF+-MS: *m/z* = 918.21 [(M + 1)$^+$]; Anal. ($C_{40}H_{41}N_9O_9P_2S_2$·2acetone) C, H, N.

***N*^{1}-[[[5″-(Phenylthio)phosphoryl]oxyethoxy]methyl]-5′-*O*-phosphoryl-2′,3′-*O*-isopropylidene-8-azidoinosine (9c).** A solution of **8c** (120 mg, 0.13 mmol) and isoamyl nitrite (263 μL, 1.95 mmol) in pyridine-AcOH−Ac_2O (2:1:1, v/v, 4 mL) was stirred at room temperature for 8 h. After the reaction mixture was evaporated (at <30 °C), the residue was dissolved with H_3PO_2 (133 μL, 2.6 mmol) and Et_3N (181 μL, 1.3 mmol) in pyridine (3 mL), and the resulting solution was stirred for 11 h at room temperature. The purification of **9c** was same as the shown in **9a** and **9c** was offered as a triethylammonium salt. The compound was purified again using same column eluting with MeCN/TEAA buffer (pH 7.0) (96 mg, 84%). ^{1}H NMR (500 MHz, D_2O) δ 1.42, 1.65 (each s, each 3H, 2 × CH_3), 3.84 (m, 2H, $H_{5'}$), 4.11 (m, 4H, CH_2O, CH_2OP), 4.51 (m, 1H, $H_{4'}$) 5.18 (dd, 1H, $J_{H3',H4'}$ = 3.0, $J_{H2',H3'}$ = 6.5 Hz, $H_{3'}$), 5.32 (dd, 1H, $J_{H2',H1'}$ = 4.5, $J_{H3',H2'}$ = 6.5 Hz, $H_{2'}$), 5.42 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''a}$), 5.55 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''b}$), 6.09 (d, 1H, $J_{H1',H2'}$ = 4.5 Hz, $H_{1'}$), 7.20−7.42 (m, 5H, Ar H), 8.19 (s, 1H, H_2); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ 3.01(s), 17.65(s). HRMS (TOF, negative) for $C_{22}H_{27}N_7O_{12}P_2S$: Calcd, 675.0914 [(M − 1)$^-$]; found, 675.0956.

***N*^{1}-[(5″-*O*-Phosphorylethoxy)methyl]-2′,3′-*O*-isopropylidene-5′-*O*-phosphoryl-8-azidoinosine 5′,5″-Cyclicpyrophosphate (10c).** A solution of **9c** (29 mg, 33 μm) in pyridine (5 mL) was added slowly over 20 h, using a syringe pump, to a mixture of I_2 (175 mg) and MS 3 Å (1.75 g), in pyridine (50 mL) at room temperature in the dark. After purification as described in **10a**, **10c** was obtained as a triethylammonium

salt. The compound was purified again using same column eluting with MeCN/TEAB buffer (pH 7.5) (22 mg, 87%). ^{1}H NMR (500 MHz, D_2O) δ 1.45, 1.61 (each s, each 3H, 2 × CH_3), 3.76−3.94 (m, 6H, CH_2O, $H_{5'}$, CH_2OP), 4.55 (m, 1H, $H_{4'}$), 5.30 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''a}$) 5.41 (dd, 1H, $J_{H4',H3'}$ = 2.0 Hz, $J_{H2',H3'}$ = 6.0 Hz, $H_{3'}$), 5.89 (d, 1H, $J_{H2',H3'}$ = 6.0 Hz,$H_{2'}$), 5.94 (d, 1H, $J_{H1''a,H1''b}$ = 10.5 Hz, $H_{1''b}$), 6.13 (s,1H, $H_{1'}$), 8.45 (s, 1H, H_2); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ −9.99 (d, $J_{P,P}$ = 10.0 Hz), −10.72(d, $J_{P,P}$ = 10.0 Hz). HRMS (TOF, negative) for $C_{16}H_{21}N_7O_{12}P_2$ Calcd, 564.0723 [(M − 1)$^-$]; found, 564.0637.

***N*^{1}-[(5″-*O*-Phosphorylethoxy)methyl]-5′-*O*-phosphoryl-8-azidoinosine 5′,5″-Cyclicpyrophosphate (2c)**. The same procedure for the deprotection of **10a** was applied to **10c** (12 mg, 15.6 μmol) in 60% HCOOH (5 mL) to give the target molecule **2c** (10.5 mg) in 93% yield. ^{1}H NMR (500 MHz, D_2O) δ 3.75−3.85 (m, 2H, CH_2O), 3.93−9.98 (m, 2H, CH_2OP), 4.02−4.05 (m, 1H, $H_{5a'}$), 4.27−4.31(m, 2H, $H_{5b'}$, $H_{4'}$), 4.85 (1H, m, $H_{3'}$), 5.26 (d, 1H, $J_{H1''a,H1''b}$ = 11.5 Hz, $H_{1''a}$), 5.68(dd, 1H, $J_{H1',H2'}$ = 4.5 Hz, $J_{H3',H2'}$ = 5.0 Hz, $H_{2'}$), 5.87(d, 1H, $J_{H1',H2'}$ = 4.5 Hz, $H_{1'}$), 5.98 (d, 1H, $J_{H1''a,H1''b}$ = 11.5 Hz, $H_{1''b}$), 8.45 (s, 1H, H_2); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ −9.72 (d, $J_{P,P}$ = 10.1 Hz), −10.10 (d, $J_{P,P}$ = 10.1 Hz); HRMS (TOF, negative) for $C_{13}H_{17}N_7O_{12}P_2$ Calcd, 524.0410 [(M − 1)$^-$]; found, 524.0344.

***N*^{1}-[(5″-*O*-Phosphorylethoxy)methyl]-5′-*O*-phosphoryl-8-aminoinosine 5′,5″-Cyclicpyrophosphate (2d).** The solution of **10c** (10 mg, 13 μmol) in ethanol (5 mL) was stirred hydrogen in the presence of 10% Pd/C at room temperature for 8 h, then the catalyst was filtered and the solution was evaporated under reduced pressure. The purification of the residue was performed at the same procedure as compound **10c** to give **10d** in 99% yield (9.5 mg). Compound **10d** was deprotected with 60% formic acid solution at room temperature and purified by twice HPLC on C18 reverse column eluting with MeCN/TEAA (pH 7.0) and MeCN/TEAB (pH 7.5) buffer, respectively, to give the target molecule **2d** (8 mg. 90%.) ^{1}H NMR (500 MHz, D_2O) δ 3.74−3.81 (m, 2H, CH_2O-), 3.91−9.93 (m, 2H, CH_2OP), 4.02−4.04 (m, 1H, $H_{5a'}$), 4.27−4.31 (m, 2H, $H_{5b'}$, $H_{4'}$), 4.85 (1H, m, $H_{3'}$), 5.23 (d, 1H, $J_{H1''a,H1''b}$ = 11.0 Hz, $H_{1''a}$), 5.70 (t, 1H, $J_{H1',H2'}$ = 5.0 Hz, $J_{H3',H2'}$ = 5.0 Hz, $H_{2'}$), 5.84 (d, 1H, $J_{H1',H2'}$ = 5.0 Hz, $H_{1'}$), 5.98 (d, 1H, $J_{H1''a,H1''b}$ = 11.0 Hz, $H_{1''b}$), 8.34 (s, 1H, H_2); ^{31}P NMR (D_2O 81 MHz, decoupled with ^{1}H) δ −9.75 (d, $J_{P,P}$ = 10.5 Hz), −9.90 (d, $J_{P,P}$ = 10.5 Hz); HRMS (TOF, positive) for $C_{13}H_{19}N_5O_{12}P_2$ Calcd, 500.0505 [(M + 1)$^-$]; found, 500.0834.

***N*^{1}-[(5″-*O*-Phosphorylethoxy)methyl]-5′-*O*-phosphoryl-8- chloroinosine 5′,5″-Cyclicpyrophosphate (2e)** was prepared by followed the known procedure.[30]

Pharmacology. Cell Culture. Jurkat T-lymphocytes (clone JMP and #E2) were cultured as described.[35,36] In brief, cells were cultured in RPMI 1640 supplemented with Glutamax I, 25 mM HEPES, 100 units/mL penicillin, 50 μg/mL streptomycin (both clones), 7.5% (v/v) newborn calf serum (clone JMP), 10% (v/v) fetal calf serum, 1 mM sodium pyruvate, 50 μg/mL hygromycin and 400 μg/mL G418-Sulfat (clone #E2).

Loading of Cells with Fura2/AM. The cells were loaded with Fura-2/AM as described[32] and kept in the dark at room temperature until use.

Determination of $[Ca^{2+}]_i$ in Intact Cells. $[Ca^{2+}]_i$ was measured in the presence of 1 mM extracellular Ca^{2+} in Jurkat T-lymphocytes using Fura2/AM as described[35] except that a F-2000 spectrofluorimeter (Hitachi) was used in the ratio mode. A modified version of the Ca^{2+} free/Ca^{2+} readdition protocol[37] was used. For the latter, 2.4 × 10^7 Jurkat cells were pelleted for 5 min at 500*g*, and the supernatant was removed. Cells were washed in extracellular solution composed of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 0.1% bovine serum albumin, 15 mM HEPES, pH 7.4, and resuspended in 1 mL of extracellular solution supplemented with 4 μM Fura2/AM. Cells were incubated for 30 min at 37°C, washed once and resuspended in extracellular solution at 2 × 10^6 cells/mL. Aliquots of 2 mL were kept in the dark at room temperature until use. Before each measurement, cells were washed twice in extracellular solution without $CaCl_2$. Changes in Fura2 fluorescence were measured using a Hitachi F-2000 spectrofluorometer (alternating excitation at 340 and 380, emission 495 nm). Intracellular $[Ca^{2+}]$ was calculated after calibration using 0.1% Triton X-100 to obtain the maximal ratio and EGTA/Tris (8 mM/60 mM) to obtain the minimal ratio.

Determination of $[Ca^{2+}]_i$ in Permeabilized Cells. Jurkat T cells were permeabilized by saponin and experiments were done exactly as described.[27] In brief, cells were exposed to saponin in an intracellular buffer (20 mM HEPES, 110 mM KCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 10 mM NaCl, pH 7.2) in the absence of extracellular Ca^{2+}. Then, saponin was removed by repeated wash procedures, and the cells were finally resuspended in intracellular buffer. After a 2 h period on ice to allow for resealing of intracellular stores, the stores of permeabilized cells were reloaded upon addition of ATP, and an ATP-regenerating system consisting of creatine phosphate and creatine kinase.[35] $[Ca^{2+}]$ was monitored fluorimetrically by addition of Fura2/free acid as described above for intact cells. When the Ca^{2+} stores were refilled, cIDPRE or cADPR were added. Each experiment was calibrated using excess $CaCl_2$ and EGTA/Tris.

Ratiometric Ca^{2+} Imaging. All procedures were carried out exactly as described in detail in previous publications.[36,38,39] In brief, we used an Improvision imaging system (Heidelberg, Germany) consisting of a monochromator system (Polychromator IV, TILL Photonics, Graefelfing, Germany) and a gray scale CCD camera (type C4742-95-12ER; Hamamatsu, Enfield, United Kingdom; operated in 8-bit mode) built around a Leica DM IRB2 microscope at 100-fold magnification. Digital confocal images were obtained by mathematical deconvolution based on the point-spread function using the nearest-neighbor algorithm (Openlab software v3.0.9, Improvision, Heidelberg).

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Appendix

Abbreviations: Ac, acetyl; cADPR, cyclic adenosine 5′-diphosphoribose; cIDPR, cyclic inosine 5′-diphosphoribose; cADPcR, cyclic adenosine 5′-diphosphocarboribose; cIDPRE, N^1-[(5″-*O*-phosphorylethoxy)methyl]-5′-*O*-phosphorylinosine 5′,5″-cyclicpyrophosphate; NAD^+, nicotinamide adenosine dinucleotide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ^{1}H NMR, proton nuclear magnetic resonance; ^{13}C NMR, carbon-13 nuclear magnetic resonance; ^{31}P NMR, phosphorus nuclear magnetic resonance; HMBC, heteronuclear multiple bond correlation; HRFAB MS, high-resolution fast atom bombardment mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; TOF, time-of-flight; HPLC, high performance liquid chromatography; $InsP_3$, inositol trisphosphate, 3 Å MS, 3 Å molecular sieve, THF, tetrahydrofuran; Py, pyridine; TPSCl, triisopropylbenzenesulfonyl chloride; PSS, cyclohexylammonium *S*,*S*-diphenyl phosphorodithioate; TBDMSCl, *tert*-butyldimethylsilyl chloride; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate; TBAF, tetrabutylammonium fluoride; TMS, tetramethyl silicane; UV, ultraviolet spectrometry.

Supporting Information Available: Elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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