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FULL PAPER

Calcium mobilizing behaviors of neutral cyclic ADP-ribose mimics which integrate the modifications of nucleobase, northern ribose and pyrophosphate

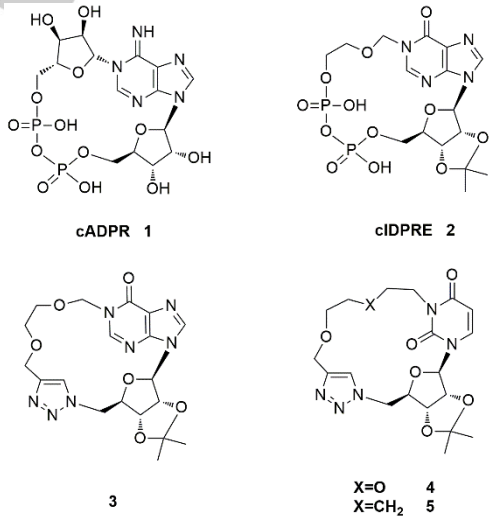
Xuan Wang, Xiaoyan Zhang, Kehui Zhang, Jianxing Hu, Zhenming Liu, Hongwei Jin*, Liangren Zhang*[a], and Lihe Zhang

Abstract: Cyclic adenosine diphosphate ribose (cADPR) is an endogenous Ca^{2+} mobilizer involved in diverse cellular process. Mimics of cADPR play crucial role in investigating the molecular mechanism of cADPR mediated signaling pathway. Herein, a mimic of cADPR (**3**) was firstly synthesized, in which neutral triazole moiety and ether linkage were introduced to substitute pyrophosphate and northern ribose, respectively. The pharmacological activities in Jurkat cells indicated that this mimic is capable of permeating plasma membrane and inciting Ca^{2+} release from endoplasmic reticulum (ER) via ryanodine receptors (RyRs) and trigger Ca^{2+} influx. Furthermore, uridine moiety was introduced and novel cADPR mimics (**4, 5**) were synthesized. The results of biological investigation showed that these mimics also targeted to RyRs and maintained moderate Ca^{2+} agonistic activities. The results indicated that the neutral cADPR mimics had the same targets to motivate Ca^{2+} signaling.

Introduction

Cyclic ADP-ribose (cADPR, **1**), an endogenous nucleotide synthesized from nicotinamide adenine dinucleotide (NAD^+) by ADP-ribosyl cyclases in cells, was firstly discovered in 1987 by Lee and colleagues as a potent Ca^{2+} releasing second messenger.[1] Recently, cADPR has drawn a great deal of attention because of its potent calcium-mobilizing activities in many cellular processes, such as fertilization, insulin secretion, lymphocyte activation and proliferation.[2] CD38, the main mammalian ADP-ribosyl cyclase, regulates both the formation and hydrolysis of cADPR.[3] It has been shown that cADPR elicits Ca^{2+} release from endoplasmic reticulum (ER) through the ryanodine receptors (RyRs) and induces Ca^{2+} influx in many cell types. Some accessory proteins, such as calmodulin (CaM), FK506-binding protein 12.6 (FKBP12.6), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), *etc*., have been reported to regulate the interaction of cADPR and RyRs.[4]

To explore the molecular mechanism inherent in the cADPR-mediated signaling pathway, a series of cADPR analogues including the modification on ribose, nucleobase, and pyrophosphate have been synthesized. Northern ribose is the most widely investigated moiety among the cADPR analogues,[5] and the research results indicated that the northern ribose was tolerant of chemical modification and relative to plasma membrane permeability. For instance, cIDPRE (**2**) and its derivatives, analogues of cyclic inosine diphosphate ribose (cIDPR) in which the northern ribose is replaced by ether linkage, are membrane permeable Ca^{2+} agonists.[6]



Scheme 1. Structures of cADPR (**1**), cIDPRE (**2**) and the novel cADPR mimics (**3-5**).

Another main factor interrupting the permeability and activity of cADPR is the strong negative charge at the pyrophosphate moiety. Up to now, only a few structural analogues modified on the pyrophosphate moiety have been synthesized, and most of these are agonists. For example, cATPR, with a triphosphate bridge, was proven to be considerably more potent than cADPR in inducing Ca^{2+} release from rat brain microsomes.[7] And agonistic activity was retained when the pyrophosphate linkage was shrunk to a monophosphate.[8] Sulfur and selenium-substituted cyclic

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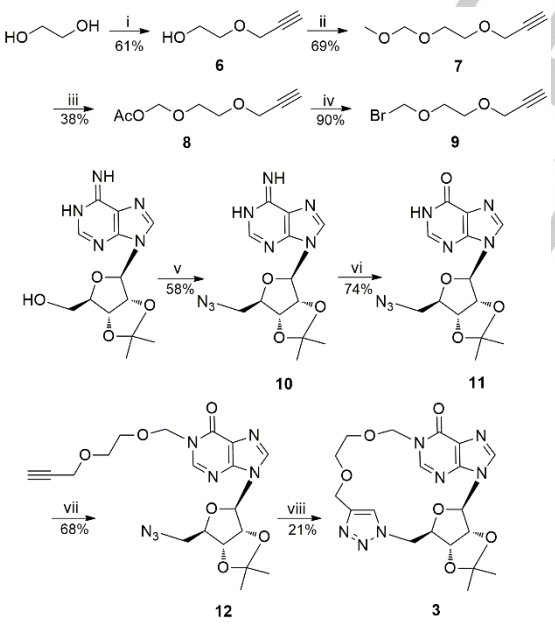
pyrophosphate cIDPRE derivatives also lowered the agonistic activity.[6d, 9] Recently, Potter and colleagues have reported a series of triazole analogues of cIDPR, albeit weak, retained the ability to activate global cytosolic Ca^{2+} release in sea urchin egg homogenate.[10] These findings raised a question that whether cADPR mimics with the disappearance of the pivotal pyrophosphate or even more structural diverse mimics still possess similar mechanism to regulate the cADPR-involved Ca^{2+} signaling pathway.

To investigate the effect of pyrophosphate moiety on the mechanism of Ca^{2+} mobilization, herein, a neutral cADPR mimic (**3**) was synthesized, in which the modifications of pyrophosphate and ribose were integrated, and the isopropylidene group at southern ribose was maintained since our previous work showed it did not affect activity to any great extent.[11] Furthermore, uridine moiety was firstly introduced and novel cADPR mimics (**4**, **5**) were synthesized (Scheme.1). The biological studies were performed in Jurkat cells. The findings of this research will help us to estabish the structure-activity relationship of cADPR analogues in detail and provide stable membrane-permeable probes to investigate cADPR-regulated Ca^{2+} signaling pathway.

Results

Synthesis of neutral cADPR mimics (3-5).

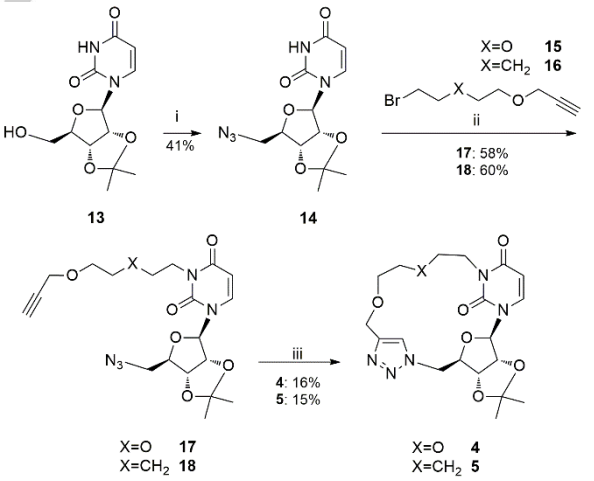
The synthetic routes adopted for compounds **3**-**5** have two main steps: (i) introduction of the northern chain onto N^1- or N^3-position, which produces intermediates **12**, **17** and **18**; (ii) cyclization of these intermediates to yield the target compounds **3**-**5** (Scheme. 2-3).



Scheme 2. Synthetic route of compound **3**. Reagents and conditions: (i) propargyl bromide, KOH, H_2O, 0 °C - rt, 18 h; (ii) Methylal, P_2O_5, DCM, 24 h, 69%; (iii) Ac_2O, $BF_3{\cdot}OEt_2$, 0°C, 2 h; (iv) TMSBr, DCM, 0 °C - reflux,18 h; (v) DPPA, DBU, 1,4-dioxane, rt, 3 h, then NaN_3, TBAI, 15-crown-5, reflux, 4 h; (vi) $NaNO_2$, AcOH, rt, 24 h; (vii) compound **9**, DBU, DCM, -10 °C; (viii) CuI, DIPEA, THF, reflux, 16 h.

Briefly, compound **6**[12] was mixed with P_2O_5 and dimethoxymethane in ice-water bath for 24 h to afford **7** (Scheme. 2).[13] Compound **7** was acetylated by acetic anhydride in the presence of boron trifluoride−diethyl etherate to generate compound **8**, which was then reacted with TMSBr to produce **9**. It is worth mentioning that the reaction time of **8** should be handled strictly to prevent byproducts. Compound **9** can be used directly for next step without purification. The N^1-substitution product **12** was carried out regioselectively by the reaction of **9** and 5'-deoxy-5'-azido-2',3'-O-isopropylidene inosine (**11**)[14] in the yield of 68%, and the O^6-alkylation by-product was observed in 13% yield. After the intramolecular click reaction catalyzed by CuI/DIPEA/THF, neutral cADPR mimic **3** was afforded. The structure was characterized by ^1H, ^{13}C-NMR and HRMS. In ^1H-NMR spectroscopy, the singlet at 7.19 ppm was observed, which indicated the generation of a 1,4-disubstituted-1,2,3-triazole (Figure. S1).

N^3-substitution of the uridine derivative was also performed similarly by the reaction of 5'-deoxy-5'-azido-2',3'-O-isopropylidene uridine (**14**)[14] and compound **15**[15] or **16** in the presence of K_2CO_3, and uridine derivatives **17** or **18** were afforded, respectively (Scheme. 3). Subsequently, compound **17** or **18** was converted to **4** or **5** using similar reaction condition as described above. In addition, we tried to remove isopropylidene group, however, treatment of **3**-**5** with aqueous HCOOH led to the rapid decomposition of the starting material or get product in very low yield.



Scheme 3. Synthetic route of compounds **4** and **5**. Reagents and conditions: (i) DPPA, DBU, 1,4-dioxane, rt, 3 h, then NaN_3, TBAI, 15-crown-5, reflux, 4 h; (ii) **15** or **16**, K_2CO_3, DMF : acetone = 1:1, 55 °C, 4 h; (iii) CuI, THF, DIPEA, reflux, 24 h.

Compounds 3-5 induce cytosolic Ca^{2+} increase in Jurkat cells.

The ability of the novel mimics to induce Ca^{2+} release in Jurkat cells was investigated. The results showed that compounds **3**-**5** were all Ca^{2+} agonists. Among them compound **3** produced strongest Ca^{2+} elevations (Figure. 1a). Compound **4** triggered impotent Ca^{2+} increase, indicating the uridine base would mitigate the ability of activating Ca^{2+} release, while compound **5** produced much weaker Ca^{2+} increase even at high concentration. Therefore, we chose compounds **3** and **4** for further investigation of biological activity. Both of **3** and **4** were found to agonize Ca^{2+} signaling in a dose-dependent manner under the presence or absence of extracellular Ca^{2+} (Figure. 1b-1e). The cytosolic Ca^{2+} increase observed in the presence of extracellular Ca^{2+} was more pronounced than that in the absence of extracellular Ca^{2+} (Figure. 1b vs 1c; 1d vs 1e), suggesting that Ca^{2+} influx should also be contributed.

Compounds 3 and 4 induce cytosolic Ca^{2+} increase from ER Ca^{2+} pools via RyRs.

We determined whether compounds **3** and **4** had the same targets as cADPR to trigger Ca^{2+} release. Ample evidence indicates that cADPR targets RyRs on the ER membrane in many cell types. Pretreating cells with thapsigargin (TG, 10 μM), a specific SERCA inhibitor, rendered less pronounced Ca^{2+} increase in the absence of external Ca^{2+}, consistent with the fact that cADPR induce Ca^{2+} release from the ER pools (Figure. 2a). Pretreatment with a RyR antagonist, ryanodine (20 μM), or cADPR antagonist, 8-Br-cADPR (100 μM), or RyR2/RyR3 double knockdown[16] significantly inhibited compound **3**-induced Ca^{2+} increase in Jurkat cells (Figure. 2b, 2c, S4a). Similarly, compound **4**-induced Ca^{2+} release was also inhibited by these antagonists or RyRs knockdown (Figure. 2e, 2f, S4b), and even be abolished by TG in Jurkat cells (Figure. 2d). In summary, these results demonstrate that compounds **3** and **4** evokes Ca^{2+} release via RyRs.

Mitochondria participate in compound 3-induced Ca^{2+} release.

TG was not able to completely abolish compound **3**-induced Ca^{2+} release in the absence of extracellular Ca^{2+} (Figure. 2a), which indicated that **3** might partially increase other Ca^{2+} pools in Jurkat cells. For this reason, we explored whether the mitochondrial or acidic Ca^{2+} stores participate in compound **3**-induced Ca^{2+} release. Pretreatment with 2 μM FCCP[17], a mitochondrial uncoupler, could lower compound **3**-invoked Ca^{2+} increase (Figure. 3a). However, lysosomal inhibitors, 50 μM GPN[18] and 10 mM NH_4Cl[19], failed to change the Ca^{2+} signaling (Figure. 3b, S5). This evidence indicated that compound **3**-induced Ca^{2+} signaling was related to mitochondria, not lysosomes.

Compounds 3-5 have similar conformation as cADPR.

Three-dimensional conformation superimposition was performed to assess the conformation similarity between cADPR and compounds **3**-**5**. All mimics were placed in similar positions with cADPR after minimization, and showed pronounced overlap in the southern ribose sugar and pyrophosphate backbone (Figure. 4). These findings suggest that the triazole moiety can mimic the pyrophosphate moiety to some extent.

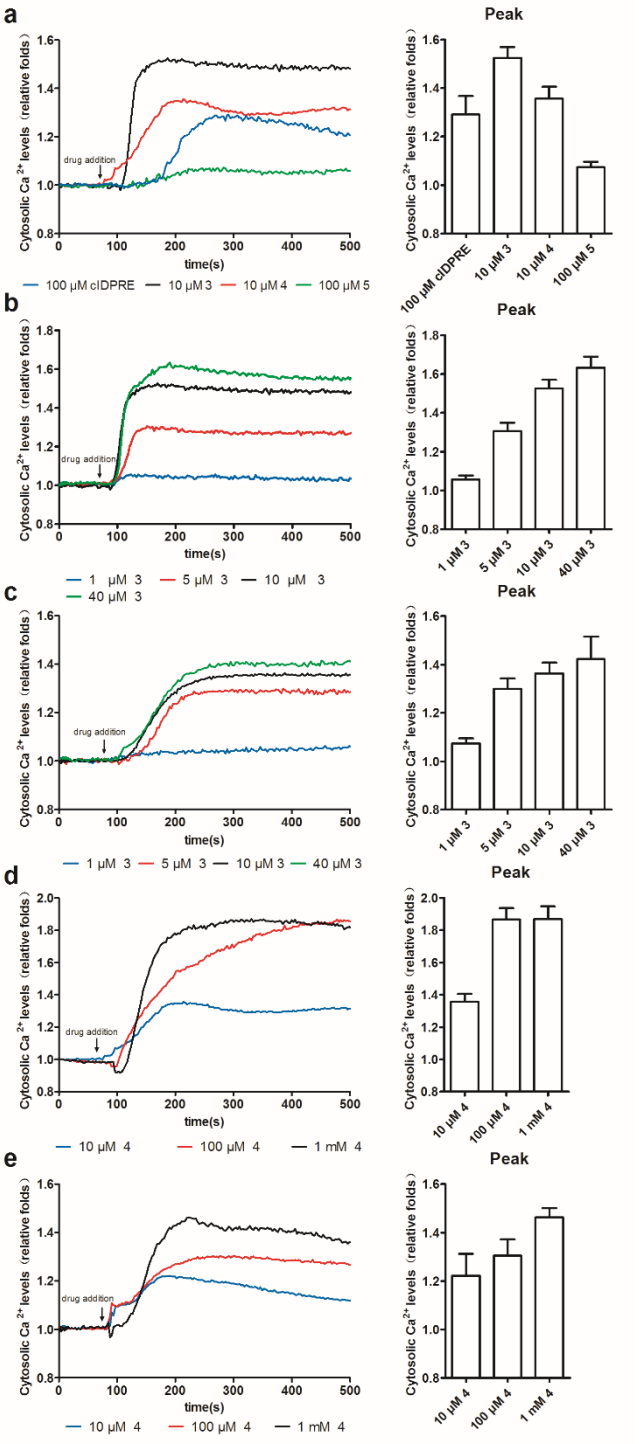


Figure 1. Compounds **3**-**5** are cell permeable cADPR agonists. (**a**) Compound **3** (10 μM) induced strongest cytosolic Ca^{2+} increase among cIDPRE (100 μM), **4** (10 μM) and **5** (100 μM) in human Jurkat cells in the presence of extracellular Ca^{2+}. Concentration response relationship of **3** or **4**-induced cytosolic Ca^{2+} increase in Jurkat cells in the presence (**b**, **d**) or absence (**c**, **e**) of extracellular Ca^{2+}. Date are the mean ± S.D. (n=3, 20-40 cells in each independent experiment).

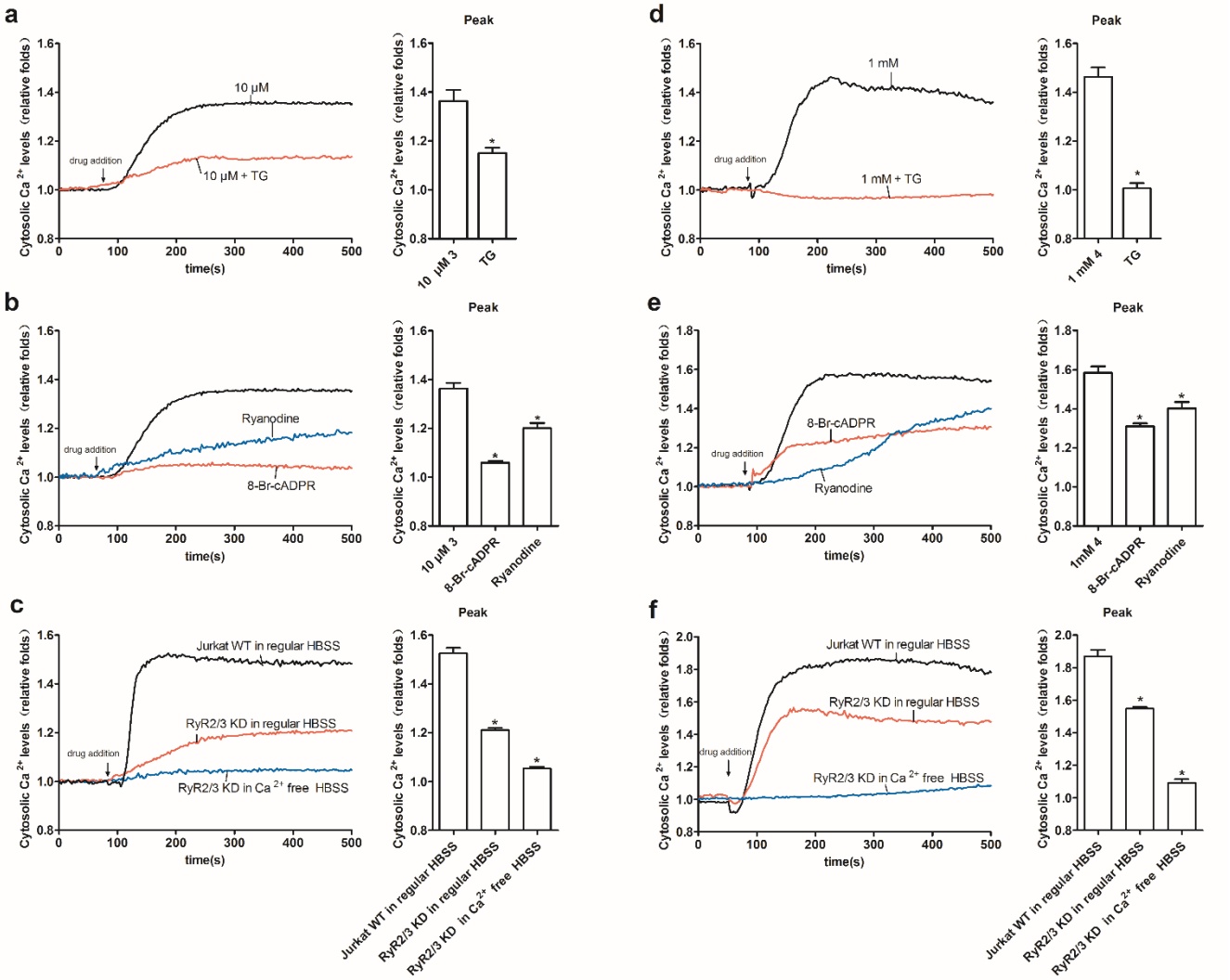


Figure 2. The requirement of RyRs for compounds **3** and **4**-triggered Ca^{2+} release. Pretreatment with TG (1 μM) markedly reduced **3**-induced Ca^{2+} increase(**a**) or even blocked **4**-induced Ca^{2+} increase(**d**) in the absence of extracellular Ca^{2+}. Compound **3** (10 μM) (**c**) or **4** (1 mM) (**f**) induced cytosolic Ca^{2+} increase was significantly inhibited by ryanodine (20 μM) or 8-Br-cADPR (100 μM) in the absence of extracellular Ca^{2+}. RyR2/RyR3 double knockdown in Jurkat cells markedly inhibited. Date are the mean ± S.D., n=3 (20-40 cells in each independent experiment). *, $P < 0.05$.

Discussion

The cADPR-mediated Ca^{2+} signaling pathway is involved in a wide variety of cellular processes.[2b] The molecular mechanism underlying its action remains unclear even though many active cADPR analogues have been used as probes. Chemical modifications on pyrophosphate has been less investigated than modifications on ribose and nucleobase. Pyrophosphate was considered to participate in cADPR's calcium signaling and to be the moiety that facilitates electrostatic interaction with binding protein.[20] Synthesis and study of the mechanism underlying pyrophosphate-missed mimics have been challenging.

So far, no pyrimidine-based modification has been involved since studies of major modifications have focused on the purine scaffold. Purine-simplified moiety have been used to assess the minimum structural requirement for activity, in which adenine was substituted by triazole.[21] Three-dimensional conformation analysis showed that pyrimidine-derivative and another neutral

mimic (3) had positions similar to that of cADPR, and they all had strong overlap in the southern ribose sugar and pyrophosphate backbone (Figure. 4). In this way, our investigation of pyrimidine derivative went the way we expected.

cADPR is readily hydrolyzed by CD38 NADase at the unstable N^1 link to produce ADPR, another Ca^{2+} second messenger.[22] Chemical modifications, such as cIDPR and cIDPRE, provided relatively stable analogues.[23] Incubation of compounds **3**-**5** for 18 h with CD38 NADase did not result in any metabolism (Figure. S2). Their stability suggested that the newly synthesized cADPR mimics might be valuable probes to investigate cADPR mediated Ca^{2+} signaling pathway.

cIDPRE is a cell-permeable agonist, yet its activity to induce Ca^{2+} release is still not strong enough to satisfy detailed biological investigation. In present study, with the pyrophosphate linkage changed to triazole moiety, **3** induced an immediate Ca^{2+} peak and a subsequent plateau phase, indicating that triazole could improve the potency of invoking Ca^{2+} release. Moreover, the Ca^{2+} inducing activity of **3** was much more pronounced than NPE-cADPR (Figure. S3), a photolyzing caged cADPR analogue, or cIDPRE (Figure. 1a). The result might be contributed by the increased permeability of the neutral mimic. Unlike **3**-induced Ca^{2+} signaling, NPE-cADPR induced Ca^{2+} signaling at 10 μM was found to decrease after a peak, and we reasoned that the small quantity of cADPR released by NPE-cADPR after UV photolysis was too slight to invoke the global Ca^{2+} increase and might be immediately hydrolyzed in Jurkat cells.

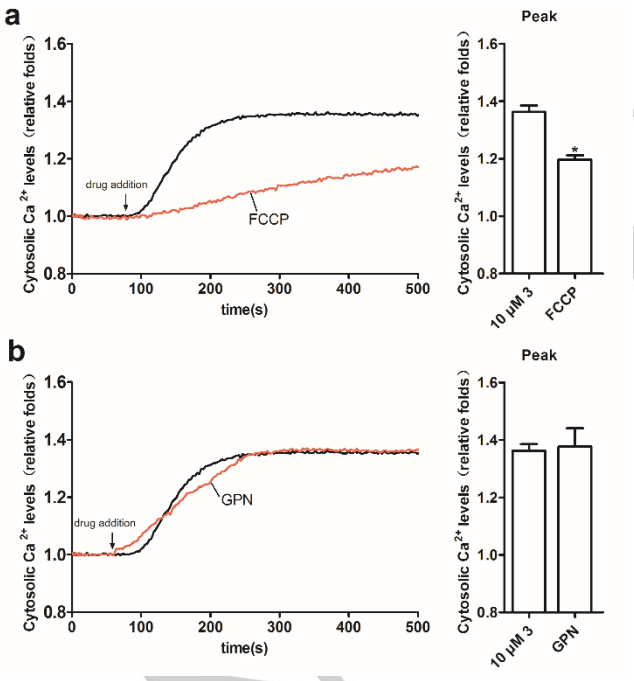


Figure 3. The requirement of mitochondria for compound **3**-triggered Ca^{2+} releases. Compound **3** (10 μM) induced Ca^{2+} increase was inhibited by FCCP (2 μM) pretreatment in the absence of extracellular Ca^{2+} (**a**). Pretreatment of cells with GPN (50 μM) did not affect compound **3** (10 μM) induced Ca^{2+} increase in the absence of extracellular Ca^{2+} (**b**). Date are the mean ± S.D., n=3-5 (20-40 cells in each independent experiment). *, $P < 0.05$.

The biological activity of **4** and **5** showed that the uridine substitution decreased agonistic activity (Figure. 1a), which indicated the importance of purine of cADPR mimics. Moreover, the oxygen atom of the northern ether chain is important since **5** activated less pronounced Ca^{2+} release than **4**, as mentioned above.[24]

cADPR regulates Ca^{2+} release directly or indirectly via RyRs. Here we found that **3** and **4** also regulated similar Ca^{2+} signaling pathway which involved Ca^{2+} release from ER via RyRs and concomitant Ca^{2+} influx (Figure. 2, S4). The results indicated that the major changing of structure, especially the substitution of pyrophosphate moiety by triazole did not affect cADPR mimics to motivate Ca^{2+} signaling via RyRs. Unexpectedly, **3** and **4** tended to trigger Ca^{2+} influx, since the induced Ca^{2+} signaling still existed in RyR2/RyR3 double knockdown Jurkat cells under the presence of extracellular Ca^{2+} (Figure. 2c and 2f). Moreover, unlike cADPR and other known cADPR agonists, the Ca^{2+} stimulation caused by **3** was inhibited by FCCP-induced mitochondrial depolarization (Figure. 3a). Previous studies also proved that agonists of IP_3Rs induced Ca^{2+} increase were inhibited by FCCP in oligodendrocytes, HeLa and smooth muscle cells.[17, 25] Due to the fact that RyRs and IP_3Rs are homogenous channels on ER, it follows that both might be related to mitochondrial activity. Mitochondria are important Ca^{2+}-storing organelles closely associated with ER and can rapidly sequester Ca^{2+},[26] yet little is known about their effect on cADPR/RyRs/Ca^{2+} pathway. Further research on cADPR mimics, RyRs and mitochondria is still underway.

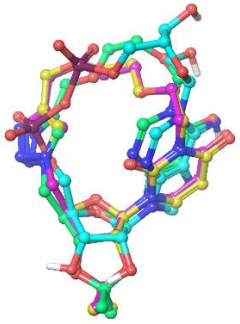


Figure 4. Superimposition of minimized conformations of cADPR and compounds **3**-**5**. cADPR is shown in light blue, compound **3** in green, compound **4** in purple and compound **5** in yellow.

Conclusions

In present study, we synthesized three novel neutral cADPR mimics (**3**-**5**) and then explored their pharmacological activities in Jurkat cells. The agonistic activities of cADPR mimics might result from the consistence of conformations even if the structures were simplified both in the nucleobase and pyrophosphate regions (Figure. 4). Besides, the nucleobase mimics **4** and **5** showed that the uridine modification of adenine decrease agonistic activity and the purine ring is important for maintaining high agonistic activity. Moreover, it showed that **3** and **4** invoked Ca^{2+} release via RyRs. Highly polar phosphate groups of adenosine nucleotide-based

second messengers - such as cADPR, NAADP and ADPR - probably correspond to positively-charged or electron-poor residues in their binding counterpart, an interaction often presumed to be critical for their activity.[20] These findings proved that the cADPR mimics with the neutral modification to pyrophosphate keep most of the same targets as cADPR and cIDPRE in regulating the Ca^{2+} pathway. These findings provided a new perspective for understanding the cADPR signaling pathway. Considering the cell selectivity of cADPR mimics in mobilizing calcium signaling,[27] the biological behaviors of these neutral mimics in other cell systems are underway.

Experimental Section

Agents and materials: NPE-cADPR[28], cIDPRE[29] and 8-Br-cADPR[30] were synthesized as detailed previously. Other chemicals purchased from Beijing J&K Chemical Ltd. and Shanghai Sigma Trading Co Ltd.. All anhydrous reagents and solvents were obtained by dehydration according to the standard methods before use. DCM was distilled from CaH_2. THF was freshly distilled from sodium. Other reagents and solvents were obtained locally and of analytical grade. Recombinant soluble mouse CD38 and ADP-ribosyl cyclase from *Aplysia californica* were supplied by Zhao, Y. J. (Shenzhen Graduate School of Peking University, China).

Synthesis of 3-[2-(methoxymethoxy)ethoxy]-1-propyne (7): To the solution of **6**[12] (2 g, 20 mmol) and dimethoxymethane (4 mL, 45 mmol) in DCM (4 mL) was added P_2O_5 (1 g, 7 mmol) at 0°C. After 24 h, DCM and $NaHCO_3$ (sat. aq.) were added, and the organic layer dried with anhydrous Na_2SO_4 and evaporated. Then the residue was applied to a column of silica gel with PE: EtOAc (8:1 v/v) to yield **7** in 69% (1.98 g) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.59 (s, 2H), 4.15 (d, *J* = 2.4 Hz, 2H), 3.64 (s, 4H), 3.30 (s, 3H), 2.40 (t, *J* = 2.4 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 96.5, 79.5, 74.6, 68.9, 66.5, 58.3, 55.1.

Synthesis of 3-[2-(acetoxymethoxy)ethoxy]-1-propyne (8): To **7** (670 mg, 4.65 mmol) in acetic anhydride (1.1mL) was added boron trifluoride−diethyl etherate (0.30 mL) at 0°C, then the solution was stirred for 2 h. The reaction mixture was diluted with DCM, washed with $NaHCO_3$ (sat. aq.) and dried with anhydrous Na_2SO_4. After evaporation the residue was applied to a column of silica gel with PE: EtOAc (7:1 v/v) to yield **8** in 38% (2.76 g) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.26 (s, 2H), 4.16 (s, 2H), 3.79-3.77 (m, 2H), 3.67-3.65 (s, 2H), 2.43 (s, 1H), 2.05 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.5, 89.2, 79.4, 74.7, 69.3, 68.7, 58.4, 20.9.

Synthesis of N^1-[(2''-propynyl) ethoxylmethyl]-5'-azido-5'-deoxy-2',3'-O-isopropylidene inosine (12): To **8** (145 mg, 0.841 mmol) in DCM (2mL) was added TMSBr (0.25 mL) at 0°C, then the solution was refluxed for 18 h. The residue was concentrated under reduced pressure to afford 3-[2-(Bromomethoxy)ethoxy]-1-propyne (**9**). Subsequently, **9** (140 mg, 0.730 mmol) was added to the solution of **11**[14] (60 mg, 0.180 mmol) and DBU (0.2 mL, 1.51 mmol) in DCM (2 mL) at 0°C. After being stirred for 4 h, the solvent was evaporated and the residue was purified by silica gel column chromatography with PE: acetone (5:1 v/v) to afford compound **12** in 68% (54 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (s, 1H), 7.91 (s, 1H), 6.05 (d, *J* = 2.7 Hz, 1H), 5.55 (s, 2H), 5.24 (dd, *J* = 6.4, 2.7 Hz, 1H), 4.94 (dd, *J* = 6.4, 3.4 Hz, 1H), 4.36 – 4.32 (m, 1H), 4.14 (d, *J* = 2.4 Hz, 2H), 3.86 – 3.81 (m, 2H), 3.66 (dd, *J* = 5.4, 3.4 Hz, 2H), 3.58 (dd, *J* = 8.3, 5.4 Hz, 2H), 2.40 (s, 1H), 1.60 (s, 3H), 1.37 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 156.6, 147.9, 147.0, 139.0, 125.1, 115.1, 90.2, 84.9, 84.3, 81.6, 79.0, 75.0, 74.6, 69.3, 68.6, 58.1, 52.1, 27.4, 25.4.

Synthesis of N^1-cyclic-ethoxylmethyl-inosine-5'-deoxy-5'-(4'''-methyl-1''',2''',3'''-triazole)-2',3'-O-isopropylidene ribose (3): To **12** (95 mg, 0.21 mmol) in THF (95 mL) was added DIPEA (79 µL, 0.460mmol). The solution was degassed with argon (30 min), CuI (52.5 mg, 0.315 mmol) added and then stirred at 65°C for 24 h. All solvents were evaporated, and the residue was purified by silica gel column chromatography eluting with PE: acetone (5:1 v/v) to afford compound **3** in 21% (20 mg)as a while amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (s, 1H), 7.79 (s, 1H), 7.20 (s, 1H), 6.11 (s, 1H), 5.93 (s, 1H), 5.23 (s, 1H), 5.09 (s, 1H), 4.97 (s, 1H), 4.88 (s, 1H), 4.55 (s, 2H), 4.39 (s, 2H), 3.94 (s, 1H), 3.73 (s, 1H), 3.64 (s, 1H), 3.53 (s, 1H), 1.65 (s, 3H), 1.37 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 156.9, 148.6, 146.2, 144.3, 139.4, 129.5, 125.2, 115.2, 88.8, 83.5, 83.0, 78.2, 74.8, 69.7, 69.3, 64.7, 48.9, 27.3, 25.7. HRMS (m/z): $[(M+H)]^+$calcd for $C_{19}H_{24}N_7O_6$, 446.1788; found, 446.1791.

Synthesis of N^3-[(2''-propynyl)ethoxylethyl]-5'-azido-5'-deoxy-2',3'-O-isopropylidene uridine (17): 15[15] (111 mg, 0.544 mmol) was added dropwise to the solution of **14** (140mg, 0.454 mmol) and anhydrous K_2CO_3 (141 mg, 1.02 mmol) in DMF (6 mL) and acetone (6 mL), and the resulting mixture was refluxed at 55°C for 4 h. The mixture was evaporated to dryness, and the residue was applied to a silica gel column chromatography eluting with PE: acetone (8:1 v/v) to yield **17** in 58% (114 mg) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.23 (d, *J* = 8.1 Hz, 1H), 5.76 (d, *J* = 8.1 Hz, 1H), 5.63 (d, *J* = 1.6 Hz, 1H), 4.97 (dd, *J* = 6.3, 1.6 Hz, 1H), 4.80 (dd, *J* = 6.3, 4.2 Hz, 1H), 4.23 (dd, *J* = 9.4, 4.9 Hz, 1H), 4.15 (dd, *J* = 10.7, 4.1 Hz, 4H), 3.71 (t, *J* = 5.9 Hz, 2H), 3.65 (s, 4H), 3.60 (d, *J* = 5.2 Hz, 2H), 2.41 (t, *J* = 2.3 Hz, 1H), 1.55 (s, 3H), 1.34 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 162.3, 150.5, 140.3, 114.8, 102.4, 95.6, 85.8, 84.4, 81.2, 79.6, 74.4, 69.8, 69.1, 67.6, 58.3, 52.2, 39.7, 27.1, 25.4.

Synthesis of N^3-cyclic-ethoxylethyl-uridine-5'-deoxy-5'-(4'''-methyl-1''',2''',3'''-triazole)-2',3'-O-isopropylidene ribose (4): To **17** (170 mg, 0.391mmol) in THF (170 mL) was added DIPEA (150 µL, 0.870mmol). The solution was degassed with argon (30 min), CuI (100 mg, 0.591 mmol) added and then stirred at 65°C for 24 h. All solvents were evaporated, and the residue was purified by silica gel column chromatography eluting with PE: acetone (5:1 v/v) to afford compound **4** in 16% (27 mg) as a while amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71 (s, 1H), 7.09 (d, *J* = 8.0 Hz, 1H), 5.75 (d, *J* = 8.0 Hz, 1H), 5.32 (dd, *J* = 6.3, 3.0 Hz, 1H), 5.24 (d, *J* = 3.0 Hz, 1H), 4.97 (dd, *J* = 14.3, 11.3 Hz, 1H), 4.85 – 4.73 (m, 2H), 4.76 – 4.56 (m, 4H), 3.92 – 3.89 (m, 2H), 3.72 – 3.51 (m, 5H), 1.56 (s, 3H), 1.37 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.5, 150.7, 146.2, 142.6, 124.5, 114.5, 102.3, 101.8, 84.2, 82.6, 82.2, 71.3, 69.4, 67.8, 65.0, 50.7, 40.9, 27.2, 25.1. HRMS (m/z): $[(M+H)]^+$calcd for $C_{19}H_{26}N_5O_7$, 436.1832; found, 436.1835.

Synthesis of N^3-[(2''-propynyl) pentyloxy]-5'-azido-5'-deoxy-2',3'-O-isopropylidene uridine (18): 16 (109 mg, 0.54 mmol) was added dropwise to the solution of **14** (140mg, 0.45 mmol) and anhydrous K_2CO_3 (141 mg, 1.02 mmol) in DMF (6 mL) and acetone (6 mL), and the resulting mixture was refluxed at 55°C for 4 h. The mixture was evaporated to dryness, and the residue was applied to silica gel column chromatography eluting with PE: acetone (8:1 v/v) to yield **18** in 60% (117 mg) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.21 (d, *J* = 8.0 Hz, 1H), 5.74 (d, *J* = 8.0 Hz, 1H) 5.61 (s, 1H), 4.95 (d, *J* = 6.4 Hz, 1H), 4.77 – 4.75 (m, 1H), 4.21 (d, *J* = 4.4 Hz, 1H), 4.09 (s, 2H), 3.86 (t, *J* = 7.4 Hz, 2H), 3.58 (d, *J* = 4.9 Hz, 2H), 3.48 (t, *J* = 6.4 Hz, 2H), 2.39 (s, 1H), 1.59 (dd, *J* = 14.7, 7.1 Hz, 4H), , 1.53 (s, 3H), 1.39 (dd, *J* = 14.7, 7.7 Hz, 2H), 1.32 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 162.5, 150.6, 139.9, 114.6, 102.4, 95.8, 85.6, 84.4, 81.7, 80.0, 74.1, 69.9, 57.8, 52.3, 41.1, 29.1, 27.3, 27.1, 25.3, 23.5.

Synthesis of N^3-cyclic-pentyloxyl-uridine-5'-deoxy-5'-(4'''-methyl-1''',2''',3'''-triazole)-2',3'-O-isopropylidene ribose (5): To **18** (173 mg, 0.40 mmol) in THF (173 mL) was added DIPEA (150 µL, 0.87 mmol). The

solution was degassed with argon (30 min), CuI (100 mg, 0.60 mmol) added and then stirred at 65°C for 24 h. All solvents were evaporated, and the residue was purified by silica gel column chromatography eluting with PE: acetone (5:1 v/v) to afford compound **5** in 15% (26 mg) as a while amorphous solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.90 (d, J = 8.0 Hz, 1H), 7.69 (s, 1H), 5.71 (d, J = 8.0 Hz, 1H), 5.61 (s, 1H), 5.22 (d, J = 6.0 Hz, 1H), 4.81 – 4.72 (m, 2H), 4.64 (dd, J = 14.8, 6.0 Hz, 1H), 4.55 (d, J = 13.2 Hz, 1H), 4.42 (d, J = 13.2 Hz, 1H), 4.26 (d, J = 6.8 Hz, 1H), 3.84 – 3.77 (m, 1H), 3.64 – 3.58 (m, 1H), 3.45 – 3.40 (m, 2H), 1.52 (s, 3H), 1.45 – 1.40 (m, 2H), 1.35– 1.31 (m, 2H), 1.30 (s, 3H), 1.22 – 1.20 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6): δ 162.5, 150.4, 145.1, 144.0, 125.1, 113.8, 100.9, 96.1, 82.6, 82.4, 79.7, 68.6, 63.9, 49.4, 27.8, 27.5, 25.9, 25.8, 23.5; HRMS(m/z): $[(M+H)]^+$calcd for $C_{20}H_{28}N_5O_6$, 434.2040; found, 434.2038.

Metabolic stability: Compounds **3-5** (50 μM) were incubated with recombinant soluble mouse CD38 (0.75 mg/mL) in buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 5 mM D-glucose, pH 7.4). Incubation was carried out as indicated at room temperature (rt) under continuous shaking. HPLC analysis of nucleotides was performed on a 250 × 4.6 mm C18-5 μm column (Agilent Technologies, Beijing, China). The separation was performed at a flow rate of 1 mL/min with water containing increasing amounts of MeCN. The gradient used for separation was (% MeCN) from 0 min (0) to 30 min (75). Compounds were detected at 254 nm.

Cell culture: Jurkat cells (from ATCC) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (PAN Seratech, Aidenbach, Germany) and 100U/mL penicillin/streptomycin (P/S). Cells were maintained at 37 °C with 5% CO_2 and 95% humidity and were passaged every 2 or 3 days.

RyR2/RyR3 shRNA lentivirus production and infection: Three shRNA oligos against human RyR2 and RyR3 were cloned into the pLKO.1 vector for expressing shRNA, then the lentivirus production and infection were performed as described previously.[16] Briefly, 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 g/mL Polybrene. Two days later, cells were selected in fresh medium containing puromycin (3 g/mL) for 3-5 days. The puromycin-resistant cells were pooled, and the knockdown efficiency was verified by quantitative real-time RT-PCR.

Calcium measurement: Jurkat cells (2 × 10^6 cells/dish) were plated in confocal dishes coated with 100 μg/mL poly-L-lysine (purchased from Sigma, P6282), respectively. Jurkat cells were incubated in serum-free medium overnight for adherence in regular medium. The adherent cells were incubated with 2 μM Fluo-2 AM (Invitrogen) in Hanks' balanced salt solution (HBSS) for 30 min in the dark at 37°C. The cells were then washed with HBSS twice and incubated in 200 μL of HBSS with or without calcium. Thereafter, the cells were put on the stage of an Olympus inverted epifluorescence microscope for measuring fluorescence intensity at 340 and 380 nm using 10× objective. Images were collected by a CCD camera every 3 s and analyzed by the MetaFluor software. Moreover, NPE-cADPR incubated cells for 20 min before images collected, followed by UV (370 nm) flash for 1 s, while cIDPRE and compounds **3**-**5** were added at 90s after images collected.

Three-dimensional conformation superimposition: The structures of compounds **3-5** were sketched in ChemBioDraw Ultra. Then Schrödinger Suite (Schrödinger, LLC, New York, NY, USA) was used to minimize compounds **3-5** and align these analogues with crystal structure of cADPR (PDB ID: 2O3Q), respectively.

Statistics: All data were averaged from at least three independent experiment. Moreover, the data represent mean ± S.D. and the Graph Pad Prism 5.01 was used for statistical analyzing. Results comparison analyzing was including two-tailed Student's t-test for two groups. Statistical significance was defined as p<0.05.

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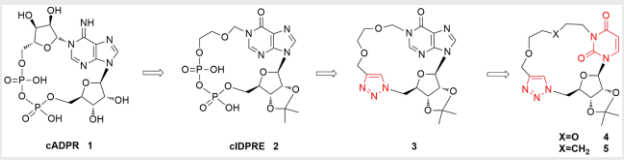

Mimics of cADPR play crucial role in investigating the molecular mechanism of cADPR-mediated calcium signaling pathway. In this study, mimics of cADPR were synthesized, in these, neutral triazole moiety, ether linkage and uridine were introduced to substitute pyrophosphate, northern ribose and nucleobase, respectively. The pharmacological activities in Jurkat cells indicated that these neutral mimics motivated the similar Ca^{2+} signaling pathway as cADPR.

Xuan Wang, Xiaoyan Zhang, Kehui Zhang, Jianxing Hu, Zhenming Liu, Hongwei Jin, Liangren Zhang*, and Lihe Zhang*

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Calcium mobilizing behaviors of neutral cyclic ADP-ribose mimics which integrate the modifications of nucleobase, northern ribose and pyrophosphate