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Synthesis and use of cell-permeant cyclic ADP-ribose

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ABSTRACT

Cyclic ADP-ribose (cADPR) is a second messenger that acts on ryanodine receptors to mobilize Ca^{2+}. cADPR has a net negative charge at physiological pH making it not passively membrane permeant thereby requiring it to be injected, electroporated or loaded via liposomes. Such membrane impermeance of other charged intracellular messengers (including cyclic AMP, inositol 1,4,5-trisphosphate and nicotinic acid adenine dinucleotide phosphate) and fluorescent dyes (including fura-2 and fluorescein) has been overcome by synthesizing masked analogs (prodrugs), which are passively permeant and hydrolyzed to the parent compound inside cells. We now report the synthesis and biological activity of acetoxymethyl (AM) and butoxymethyl (BM) analogs of cADPR. Extracellular addition of cADPR-AM or cADPR-BM to neuronal cells in primary culture or PC12 neuroblastoma cells induced increases in cytosolic Ca^{2+}. Pre-incubation of PC12 cells with thapsigargin, ryanodine or caffeine eliminated the response to cADPR-AM, whereas the response still occurred in the absence of extracellular Ca^{2+}. Combined, these data demonstrate that masked cADPR analogs are cell-permeant and biologically active. We hope these cell-permeant tools will facilitate cADPR research and reveal its diverse physiological functions.

1. Introduction

Intracellular Ca^{2+} mobilization is mediated not only by inositol 1,4,5-trisphosphate (IP_3) [1–3] but also by nictoinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPR) [4–6]. Although NAADP and cADPR are bona fide messengers [5,7,8], their role in cell physiology and the identity of their molecular targets remain hotly contested [9–11].

One impediment to the widespread investigation of cADPR is its lack of cell permeability, which has made research into the effect of cADPR itself in intact cells the preserve of labs with specialist equipment and techniques. Techniques to introduce cADPR into cells include microinjection [12] and delivery through cADPR-loaded liposomes [13]. An alternative to using cADPR itself is to use cell-permeant cADPR analogs [14–20]; however, their very hydrophilic nature makes passive permeability extremely low [21] and uptake likely depends on transporters [22–26]. Moreover, if the transporters do not exist on all cell types, as with the carrier-mediated uptake of NAADP [27], hydrophilic analogs are not a universally applicable solution.

It should be possible to make cADPR cell-permeant through chemical modification. Like other second messengers, cADPR is passively impermeant due to its size (541 Da), large polar surface area (279 square Å) and net negative charge at physiological pH [28]. Of these parameters that dictate membrane permeability, charge dominates [21]. This has been recognized for numerous charged molecules in the past and several chemical-protecting techniques can be used to mask the charge to make the molecule cell permeant [29]. Likewise, the hydrophilic contribution of hydroxyl groups can be reduced with cleavable protecting groups [30]. Once the molecule penetrates the cell, the ester is hydrolyzed by nonselective esterases regenerating the parent molecule [29,31]. A particularly successful approach is to make the methoxyester method (first reported for penicillin [32]), which has been used to great success with Ca^{2+} chelators [31], fluorescent probes [33] and second messengers including IP_3 [34–36], cyclic AMP and cyclic GMP (Li1997) and NAADP [37].

2. Materials and methods

2.1. Materials

All reagents were obtained from Sigma–Aldrich, unless otherwise stated. All water used was double deionized by a reverse osmosis filter (Purite Ltd., UK).

2.2. High-performance liquid chromatography (HPLC)

Anion-exchange HPLC was used to separate nucleotides, monitor progress of reactions and isolate synthesized compounds.

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Samples were run over a strong anion-exchange resin, AG MP-1, packed into a 3 × 150 mm borosilicate glass column (Omnifit, New Jersey, USA). Samples were injected via a Waters 600E Multisolvent Delivery System and analyzed by a Waters 2487 Dual Wavelength Absorbance Detector set at 254 nm (Waters Corporation). Elution was achieved with trifluoroacetic acid, run as an exponential gradient over 40 min [38].

2.3. Synthesis of cADPR

cADPR was synthesized from NAD as described previously [12,39]. Briefly, we incubated β-NAD 2 mM with purified ADP-ribosyl cyclase (0.1 U/mL) from *Aplysia* (provided by H.C. Lee; [39–41]) in Hepes–NaOH 50 mM, pH 7 for 3 h at room temperature. The reaction was monitored and reactants and products separated by HPLC. The cADPR peak was collected, dried with rotary evaporation under vacuum, washed three times with methanol, then three times with acetone and stored as the free acid or sodium salt at −80 °C and was routinely >95% pure by HPLC.

^{1}H NMR (D_2O, 400 MHz): δ 3.94 (m, 1H), 4.02 (m, 1H), 4.27 (m, 2H), 4.37 (m, 2H), 4.65 (m, 3H), 5.25 (t, 1H, J = 5.4 Hz), 5.97 (d, 1H, J = 5.8 Hz), 6.05 (d, 1H, J = 4.0 Hz), 8.30 (s, 1H), 8.91 (s,1H); MS (ESI) m/z 540 $(M–H)^-$.

2.4. Synthesis of butyryloxymethyl bromide

Butyric acid (5.27 g, 5.5 mL, 59.8 mmol) was added to 2 M NaOH (60 mL) in a round bottom flask and the mixture was stirred for 30 min. To this was added tetrabutylammonium hydrogen sulfate (20.4 g, 60.1 mmol) and the reaction mixture was further stirred for 30 min at room temperature. After this, the aqueous layer was extracted with dichloromethane (4 × 100 mL) and the organic layer dried over magnesium sulfate. The solution was evaporated on a rotavap and the crude product was then purified by vacuum distillation at 140 °C for 2 h to afford 7.20 g of methylene dibutyrate.

^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.93 (t, 6H, J = 7.43), 1.65 (m, 4H, J = 7.43), 2.32 (t, 4H, J = 7.43), 5.73 (s, 2H).

Methylene dibutyrate (7.20 g, 48.49 mmol), trimethylsilylbromide (7.42 g, 6.4 mL, 48.49 mmol) and zinc bromide (0.43 g, 19.10 mmol) were charged in 50 mL of round bottom flask and the reaction mixture stirred for 24 h at room temperate. Then a further portion of trimethylsilylbromide (6.4 mL, 48.49 mmol) was added and the mixture left to stir for another 24 h at room temperature. After the completion of the reaction as judged by TLC, diethyl ether (20 mL) and 1 M HCl (10 mL) were added and the reaction stirred for 15 min after which 1 M Na_2CO_3 (20 mL) was added. The reaction mixture was further stirred for 30 min and the aqueous layer extracted with ether (3 × 50 mL). The organic layer was dried over magnesium sulfate and solvent evaporated on rotavap. The crude product was purified by vacuum distillation at 120 °C to yield 4.5 g of butyryloxymethyl bromide (BM-Br) as pure product.

^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.94 (t, 3H, J = 7.43), 1.68 (m, 2H), 2.35(t, 2H, J = 7.43), 5.80 (s, 2H).

2.5. Synthesis of acetoxymethyl (AM) and butyryloxymethyl (BM) cADPR analogs

Addition of AM and BM groups to cADPR was adapted from a protocol developed by Schultz et al. [42] to esterify cAMP. In a round bottom flask, 40 mg cADPR (free acid form produced by passing through a Dowex 50 Columns, acid form), 50 μL diisopropylethylamine (DIEA) and 2.5 mL of dry acetonitrile were stirred together for 2 h under a dry argon atmosphere. The esterification reaction was initiated by addition of 100 μL acetoxymethyl bromide (AM-Br) or 100 μL BM-Br. A further 100 μL addition of AM-Br or BM-Br was made after 24 h. After stirring the reaction mixture at room temperature for 2 days, the product was vacuum dried and tested for purity by HPLC. Esterified cADPR was stored under argon at −20 °C. The solid orange–brown product was taken up in chloroform and analyzed by HPLC, phosphorous nuclear magnetic resonance, proton nuclear magnetic resonance and mass spectroscopy. The cADPR–acetoxymethyl ester (resin) was stored under argon at −80 °C. The ability to hydrolyze the cADPR back to cADPR was evaluated with alkaline hydrolysis (100 mM NaOH, 1 h, 23 °C) and incubation with a guinea pig heart homogenate (10% weight/volume).

Due to the low yields and instability of cADPR esters, as reported for the direct esterification of cAMP [29,42], we were unable to obtain enough material for NMR for absolute structural assignment. However, mass spectroscopy revealed a family of peaks separated by 73 atomic mass units indicating multiple species with varying numbers of AM groups.

2.6. Neuronal cell culture and Ca^{2+} imaging

Primary neuronal cells were cultured from p1 Wistar rat pups (Harlan UK Ltd.) as described previously [37]. Primary rat neuronal cells were loaded with fluo-3 by incubation in medium with 5 μM fluo-3-acetoxymethyl ester (Invitrogen) for 1 h at room temperature. A coverslip was mounted in a perfusion chamber and placed onto the stage of a Leica LS2000 confocal microscope supported on a vibration-isolated table. Cells were viewed through 63× water objective lens. Excitation was at 488 nm (Argon laser) and fluorescence was detected after a 515-nm long-pass filter and captured at 512 × 512 pixels.

PC12 cells (American Type Culture Collection) were cultured in DMEM containing 5% fetal bovine serum, 10% horse serum, 1% penicillin/streptomycin and 1% L-glutamine and maintained in a humidified incubator (95% O_2, 5% CO_2). Before use, PC12 cells were trypsinized and plated onto glass coverslips (25-mm diameter) coated with poly-D-lysine (0.1 mg/mL) and were differentiated with 50 ng/mL nerve growth factor. Ca^{2+} imaging experiments were performed 5 days after plating. PC12 cells were loaded with fura-2 by incubation with 4 M of its acetoxymethylester (Invitrogen) for 20 min at room temperature. Coverslips were mounted into a perfusion chamber and maintained in HBSS and imaged with a Zeiss Axiovert 2000 microscope. Fluoresence was detected with a CCD camera during alteranting 340 and 380 nm excitation after 510 long-pass filtering controlled with Metafluor 7.0 software (Molecular Devices).

3. Results and discussion

3.1. Synthesis cADPR-AM

To synthesize cADPR we incubated NAD with *Aplysia* ADP-ribosyl cyclase (Fig. 1A). The reaction is reversible [39] and reached an equilibrium of about 40% cADPR and 60% NAD at 4 h as demonstrated by anion-exchange HPLC (Fig. 1D). The cADPR peak was collected, dried down in a rotary evaporator and dissolved in double-deionized water and found to be >90% pure (Fig. 1E, left trace) and was confirmed with NMR and mass spectroscopy (Fig. 1C; see Section 2). When cADPR was reacted with the reagent acetoxymethyl bromine (AM-Br) in anhydrous acetonitrile for 48 h, it was entirely converted into products that did not bind to the anion exchange column (Fig. 1E, right trace). Analysis of this isolated peak with mass spectroscopy was consistent with cADPR with multiple AM groups (see Section 2). Further evidence for this peak being cADPR-AM was that cADPR could be obtained upon