Contents lists available at SciVerse ScienceDirect

ELSEVIER

**Biochemical and Biophysical Research Communications** 

journal homepage: www.elsevier.com/locate/ybbrc



# Synthesis and use of cell-permeant cyclic ADP-ribose

Daniel Rosen<sup>1</sup>, Duncan Bloor-Young<sup>1</sup>, James Squires<sup>1</sup>, Raman Parkesh, Gareth Waters, Sridhar R. Vasudevan, Alexander M. Lewis, Grant C. Churchill<sup>\*</sup>

University of Oxford, Department of Pharmacology, Mansfield Road, Oxford OX1 3QT, UK

#### ARTICLE INFO

Article history: Received 3 January 2012 Available online 17 January 2012

Keywords: Cyclic ADP-ribose Cell-permeant Calcium PC12 Ryanodine receptor Chemical probe

## ABSTRACT

Cyclic ADP-ribose (cADPR) is a second messenger that acts on ryanodine receptors to mobilize Ca<sup>2+</sup>. cAD-PR 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<sup>2+</sup>. 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<sup>2+</sup>. Combined, these data demonstrate that masked cADPR analogs are cell-permeant and biological functions.

© 2012 Elsevier Inc. All rights reserved.

### 1. Introduction

Intracellular Ca<sup>2+</sup> mobilization is mediated not only by inositol 1,4,5-trisphosphate (IP<sub>3</sub>) [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 cADPRloaded 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 carriermediated 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

\* Corresponding author.

E-mail address: grant.churchill@pharm.ox.ac.uk (G.C. Churchill).

<sup>1</sup> These authors contributed equally to this work.

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<sup>2+</sup> chelators [31], fluorescent probes [33] and second messengers including IP<sub>3</sub> [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.

<sup>0006-291</sup>X/\$ - see front matter  $\circledcirc$  2012 Elsevier Inc. All rights reserved. doi:10.1016/j.bbrc.2012.01.025

Samples were run over a strong anion-exchange resin, AG MP-1, packed into a  $3 \times 150$  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  $\beta$ -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.

<sup>1</sup>H NMR (D<sub>2</sub>O, 400 MHz):  $\delta$  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)<sup>-</sup>.

#### 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 ( $4 \times 100$  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.

<sup>1</sup>H NMR (CDCl<sub>3</sub>, 200 MHz)  $\delta$  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<sub>2</sub>CO<sub>3</sub> (20 mL) was added. The reaction mixture was further stirred for 30 min and the aqueous layer extracted with ether ( $3 \times 50$  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.

<sup>1</sup>H NMR (CDCl<sub>3</sub>, 200 MHz)  $\delta$  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  $\mu$ L 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  $\mu$ L acetoxymethyl bromide (AM-Br) or 100  $\mu$ L BM-Br. A further 100  $\mu$ L 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<sup>2+</sup> 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  $\mu$ M 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<sup>2+</sup> 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



**Fig. 1.** Synthesis of CADPR and its acetyoxymethyl ester (cADPR-AM). (A) Chemical structures and synthetic schemes. cADPR was synthesized enzymatically with *Aplysia* ADP-ribosyl cyclase. cADPR-AM was synthesized by dissolving its disopropylethylamine (DIEA) salt in acetonitrile and esterifying with acetyoxymethylbromine. (B) Chromatography traces showing cADPR synthesis from NAD<sup>+</sup> catalyzed by ADP-ribosyl cyclase. (C) Chromatography traces showing cADPR-AM de-esterified by either alkaline hydrolysis (1 M NaOH) or by incubation with rat heart homogenate (10% weight/ volume).



**Fig. 2.** Esterified cADPR does not release  $Ca^{2+}$  in the sea urchin egg homogenate bioassay. (A) Sea urchin egg homogenate releases  $Ca^{2+}$  in response to NAADP, cADPR and IP<sub>3</sub>. (B) cADPR releases  $Ca^{2+}$ ; whereas (C) cADPR-AM does not.

hydrolysis by either NaOH (Fig. 1F, left trace) or heart homogenate (Fig. 1F, right trace), a rich source of esterases [12,31].

# 3.2. Esterification of cADPR eliminates biological activity in sea urchin egg homogenate bioassay

The sea urchin egg homogenate is a sensitive and robust bioassay for all three Ca<sup>2+</sup>-releasing messengers, IP<sub>3</sub>, NAADP and cADPR (Fig. 3A) [5,12,28,43]. Indeed, two of these three messengers, cAD-PR [6] and NAADP [43] were discovered using this bioassay. We used this sea urchin egg homogenate bioassay to determine whether cADPR-AM had any Ca<sup>2+</sup>-releasing activity because it is sensitive to sensitive to all three Ca<sup>2+</sup>-releasing messengers (Fig. 2A) [5,6,12] and *Lytechinus pictus* sea urchin eggs contain very limited esterase activity [12,44,45] thereby minimizing the confounding effect of cADPR-AM hydrolysis. In the same batch of sea urchin egg homogenate, a supramaximal concentration of cADPR (0.5  $\mu$ M) released Ca<sup>2+</sup> (Fig. 3B), whereas cADPR-AM (100  $\mu$ M) released minimal Ca<sup>2+</sup> (Fig. 3C). These results demonstrate that addition of AM groups to the phosphates and likely the hydroxyls on cADPR eliminates its Ca<sup>2+</sup>-releasing activity. This is consistent with what is known about the importance of the phosphates and hydroxyls in cADPR for its biological activity [46,47].

# 3.3. cADPR-AM induces Ca<sup>2+</sup> increases in neuronal cells

To determine whether AM addition made cADPR cell-permeant, we tested cADPR-AM on both primary rat neurons and PC12 cells, each of which has advantages and disadvantages. While primary neurons are arguably closer to the physiological situation, PC12 cells are better characterized in regard to cADPR signaling [48,49]. PC12 cells are a pheochromocytoma stable line and widely used as a nerve cell model [50]. Importantly, for testing cADPR-AM, PC12 cells have been reported to utilize cADPR-mediated Ca<sup>2+</sup> signaling [48,49], possess functional ryanodine receptors [51], the putative cADRP receptor [5,9,11], and use cADPR as an endogenous messenger in the nitric oxide [48] and agonist-mediated [49] signaling pathways.

The addition of extracellular cADPR (100  $\mu$ M) failed to elicit an increase in intracellular Ca<sup>2+</sup> in primary neurons, even after a



**Fig. 3.** Extracellular cADPR esters induce  $Ca^{2^+}$  responses in neuronal cells. (A–C) Effects of extracellular cADPR and cADPR-AM on intracellular  $Ca^{2^+}$  in primary cultures of rat neurons. (A) Addition of extracellular cADPR-AM (100  $\mu$ M) but not cADPR (100  $\mu$ M) increases intracellular  $Ca^{2^+}$ . (B) Reducing the concentration of extracellular cADPR-AM (0.1  $\mu$ M) results in a latency in the increase in intracellular  $Ca^{2^+}$ . (C) Plot showing the relationship between cADPR ester concentration and latency to the  $Ca^{2^+}$  increase. (D–F) Effects of extracellular cADPR-AM on intracellular  $Ca^{2^+}$  in cultured PC-12 cells. (D) Addition of cADPR-AM (100  $\mu$ M) induces an intracellular  $Ca^{2^+}$  increase. (G) Addition of extracellular cADPR (100  $\mu$ M) does not affect intracellular  $Ca^{2^+}$ . (H) Concentration–response relationship for cADPR-AM and  $Ca^{2^+}$  increase (mean  $\pm$  standard error of the mean, n = 3-6).



**Fig. 4.** Pharmacological characterization of the Ca<sup>2+</sup> response to cADPR-AM in PC-12 cells. (A) Reducing extracellular Ca<sup>2+</sup> with EGTA did not reduce the Ca<sup>2+</sup> response to cADPR-AM (100  $\mu$ M). (B) Depleting intracellular endoplasmic reticulum Ca<sup>2+</sup> stores with the Ca<sup>2+</sup> pump inhibitor thapsigargin (1  $\mu$ M) eliminated the Ca<sup>2+</sup> response to cADPR-AM (100  $\mu$ M). Depleting intracellular Ca<sup>2+</sup> stores containing ryanodine receptors with (C) 100  $\mu$ M ryanodine or (D) 20 mM caffeine eliminated the Ca<sup>2+</sup> response to cADPR-AM (100  $\mu$ M). (E) Summary of the responses to cADPR and cADPR-AM in response to the various pharmacological treatments. Bars represent the mean ± standard error of the mean for *n* = 3–6. Statistical significance was evaluated with a one-way analysis of variance followed by Dunnett's multiple comparison test against the control, \**p*  $\leq$  0.05, \*\**p*  $\leq$  0.01, and \*\*\**p*  $\leq$  0.001.

10-min incubation (Fig. 3A). In contrast, in the same cells, the addition of cADPR-AM (100  $\mu$ M) elicited an increase in intracellular Ca<sup>2+</sup> within 10 s (Fig. 3A). The addition of extracellular cADPR-AM at the lower concentration of 0.1  $\mu$ M resulted in an intracellu-

lar Ca<sup>2+</sup> increase but with a longer latency between the time of addition and the time of the response (Fig. 3B). This relationship was concentration dependent and could be fit with a single-site model with an EC<sub>50</sub> of 1  $\mu$ M (Fig. 3C). In contrast to the concentration dependence observed with latency, there was no relationship with the amplitude of the response and the concentration of cAD-PR-AM and was typically all or none (Fig. 3A versus B). This may reflect that the target of cADPR is likely ryanodine receptors [5,9,11] that show an all-or-none response [1,52].

We next determined whether cADPR-AM also was effective in PC12 neurons. cADPR-AM (100  $\mu$ M) resulted in an increase in intracellular Ca<sup>2+</sup> (Fig. 3D) whereas cADPR itself did not (Fig. 3E). In contrast to primary neurons, in PC12 cells exhibited a well-defined relationship between cADPR-AM concentration and the amplitude of the Ca<sup>2+</sup> response with an EC<sub>50</sub> of 22  $\mu$ M (Fig. 4F).

# 3.4. cADPR-BM induces $Ca^{2+}$ increases in neuronal cells

When Li et al. [34] synthesized cell-permeant esters of IP<sub>3</sub>, they tested AM, propionyloxymethyl (PM) and butoxymethyl (BM) groups and found that the AM group was less effective than the PM or BM groups likely due to the balance between the rate of hydrolysis (fewer methylenes was faster) versus hydrophobicity that dictates passive permeability [34]. Therefore, we also synthesized and tested cADPR protected with BM groups. Unlike IP<sub>3</sub>-BM [34], cADPR-BM did not show a dramatically greater potency based on a concentration–response relationship based on latency, but the BM group did shift the curve slightly to the left (Fig. 3C). As the advantages of cADPR-BM were slight and the BM precursor had to be synthesized in-house rather than simply purchased, all further experiments were conducted with cADPR-AM.

# 3.5. cADPR-AM responses require intracellular and not extracellular $Ca^{2\ast}$

cADPR can activate not only ryanodine receptors but also certain plasma membrane Ca<sup>2+</sup> channels such as TRPM2 [53]. Therefore, we tested the involvement of extracellular Ca<sup>2+</sup> influx and mobilization from intracellular stores. Lowering extracellular Ca<sup>2+</sup> did not significantly affect the amplitude of the response to cADPR-AM (Fig. 4A and E). Depleting the endoplasmic reticular Ca<sup>2+</sup> stores with the intracellular Ca<sup>2+</sup> pump inhibitor thapsigargin (1  $\mu$ M; [54]) eliminated the response to cADPR-AM (Fig. 4B and E). Combined, these data are consistent with the notion that cADPR-AM is mobilizing Ca<sup>2+</sup> from the endoplasmic reticulum.

# 3.6. cADPR-AM responses require ryanodine receptors

If cADPR-AM is indeed passively permeable to cell membranes and being hydrolyzed to cADPR to elicit its  $Ca^{2+}$ -mobilizing effect, the pharmacology of the response should be consistent with that of cADPR. For these experiments we used cADPR-AM and PC12 cells. We examined the effect of depleting ryanodine receptor-containing stores on the ability of extracellular cADPR-AM to increase  $Ca^{2+}$ . Both 20 mM caffeine (Fig. 4C) and 100  $\mu$ M ryanodine (Fig. 4D) elicited large and long-lasting  $Ca^{2+}$  increases and eliminated the subsequent response to cADPR-AM (Fig. 4C–E). The sustained increases observed with caffeine and ryanodine are consistent with those reported previously with PC12 cells [48,55].

## Acknowledgments

We thank Prof. H.C. Lee (Department of Physiology, University of Hong Kong) for the gift of the ADP-ribosyl cyclase. This work was funded by a grant from the British Heart Association [Grant PG/09/056/27846].

#### References

- M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signalling, Nat. Rev. Mol. Cell Biol. 1 (2000) 11–21.
- [2] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, Nat. Rev. Mol. Cell Biol. 4 (2003) 517–529.
- [3] H. Streb, R.F. Irvine, M.J. Berridge, I. Schulz, Release of Ca<sup>2+</sup> from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate, Nature 306 (1983) 67–69.
- [4] H.C. Lee, Cyclic ADP-ribose and NAADP. A story of two calcium messengers, in: H.C. Lee (Ed.), Cyclic ADP-Ribose and NAADP Structures, Metabolism and Functions, Kluwer Academic Publishers, Boston, 2002, pp. 1–17.
- [5] H.C. Lee, Cyclic ADP-ribose and NAADP: fraternal twin messengers for calcium signaling, Sci. China Life Sci. 54 (2011) 699-711.
- [6] D.L. Clapper, T.F. Walseth, P.J. Dargie, H.C. Lee, Pyridine nucleotide metabolites stimulate calcium release from sea urchin egg microsomes desensitized to inositol trisphosphate, J. Biol. Chem. 262 (1987) 9561–9568.
- [7] A. Galione, G.C. Churchill, Cyclic ADP ribose as a calcium-mobilizing messenger, Sci. STKE 2000 (2000) pe1.
- [8] H.C. Lee, Calcium signaling: NAADP ascends as a new messenger, Curr. Biol. 13 (2003) R186-R188.
- [9] E. Venturi, S. Pitt, E. Galfré, R. Sitsapesan, From eggs to hearts: what is the link between cyclic ADP-ribose and ryanodine receptors?, Cardiovasc. Ther. (2010).
- [10] A.H. Guse, Second messenger signaling: multiple receptors for NAADP, Curr. Biol. 19 (2009) R521–R523.
- [11] A. Galione, H.C. Lee, W.B. Busa, Ca<sup>(2+)</sup>-induced Ca<sup>2+</sup> release in sea urchin egg homogenates: modulation by cyclic ADP-ribose, Science 253 (1991) 1143– 1146.
- [12] A.J. Morgan, G.C. Churchill, R. Masgrau, et al., Methods in cyclic ADP-ribose and NAADP research, in: J.W.J. Putney (Ed.), Calcium Signaling, CRC Press, Boca Raton, 2006, pp. 265–333.
- [13] E. Brailoiu, M.D. Miyamoto, Inositol trisphosphate and cyclic adenosine diphosphate-ribose increase quantal transmitter release at frog motor nerve terminals: possible involvement of smooth endoplasmic reticulum, Neuroscience 95 (2000) 927–931.
- [14] J.K. Sethi, R.M. Empson, V.C. Bailey, B.V. Potter, A. Galione, 7-Deaza-8-bromocyclic ADP-ribose, the first membrane-permeant, hydrolysis-resistant cyclic ADP-ribose antagonist, J. Biol. Chem. 272 (1997) 16358–16363.
- [15] G.K. Wagner, S. Black, A.H. Guse, B.V.L. Potter, First enzymatic synthesis of an N1-cyclised cADPR (cyclic-ADP ribose) analogue with a hypoxanthine partial structure: discovery of a membrane permeant cADPR agonist, Chem. Commun. (Camb.) (2003) 1944–1945.
- [16] X. Gu, Z. Yang, L. Zhang, et al., Synthesis and biological evaluation of novel membrane-permeant cyclic ADP-ribose mimics: N1-[(5"-0phosphorylethoxy)methyl]-5'-O-phosphorylinosine 5',5"cyclicpyrophosphate (cIDPRE) and 8-substituted derivatives, J. Med. Chem. 47 (2004) 5674–5682.
- [17] J. Xu, Z. Yang, W. Dammermann, L. Zhang, A.H. Guse, L.-H. Zhang, Synthesis and agonist activity of cyclic ADP-ribose analogues with substitution of the northern ribose by ether or alkane chains, J. Med. Chem. 49 (2006) 5501–5512.

- [18] B. Zhang, V.C. Bailey, B.V.L. Potter, Chemoenzymatic synthesis of 7-deaza cyclic adenosine 5'-diphosphate ribose analogues, membrane-permeant modulators of intracellular calcium release, J. Org. Chem. 73 (2008) 1693–1703.
- [19] T. Kirchberger, C. Moreau, G.K. Wagner, et al., 8-Bromo-cyclic inosine diphosphoribose: towards a selective cyclic ADP-ribose agonist, Biochem. J. 422 (2009) 139–149.
- [20] M. Dong, T. Kirchberger, X. Huang, et al., Trifluoromethylated cyclic-ADPribose mimic: synthesis of 8-trifluoromethyl-N(1)-[(5"-Ophosphorylethoxy)methyl]-5'-O-phosphorylinosine-5',5"-cyclic pyrophosphate (8-CF(3)-cIDPRE) and its calcium release activity in T cells, Org. Biomol. Chem. 8 (2010) 4705–4715.
- [21] A. Missner, P. Pohl, 110 years of the Meyer-Overton rule: predicting membrane permeability of gases and other small compounds, Chemphyschem. 10 (2009) 1405–1414.
- [22] L.C. Davis, A.J. Morgan, M. Ruas, et al., Ca<sup>(2+)</sup> signaling occurs via second messenger release from intraorganelle synthesis sites, Curr. Biol. 18 (2008) 1612–1618.
- [23] M. Podestà, F. Benvenuto, A. Pitto, et al., Concentrative uptake of cyclic ADPribose generated by BST-1+ stroma stimulates proliferation of human hematopoietic progenitors, J. Biol. Chem. 280 (2005) 5343–5349.
- [24] L. Guida, L. Franco, S. Bruzzone, et al., Concentrative influx of functionally active cyclic ADP-ribose in dimethyl sulfoxide-differentiated HL-60 cells, J. Biol. Chem. 279 (2004) 22066–22075.
- [25] L. Guida, S. Bruzzone, L. Sturla, L. Franco, E. Zocchi, A. De Flora, Equilibrative and concentrative nucleoside transporters mediate influx of extracellular cyclic ADP-ribose into 3T3 murine fibroblasts, J. Biol. Chem. 277 (2002) 47097–47105.
- [26] L. Franco, L. Guida, S. Bruzzone, E. Zocchi, C. Usai, A. De Flora, The transmembrane glycoprotein CD38 is a catalytically active transporter responsible for generation and influx of the second messenger cyclic ADPribose across membranes, FASEB J. 12 (1998) 1507–1520.
- [27] R.A. Billington, E.A. Bellomo, E.M. Floriddia, J. Erriquez, C. Distasi, A.A. Genazzani, A transport mechanism for NAADP in a rat basophilic cell line, FASEB J. 20 (2006) 521–523.
- [28] H.C. Lee, T.F. Walseth, G.T. Bratt, R.N. Hayes, D.L. Clapper, Structural determination of a cyclic metabolite of NAD<sup>+</sup> with intracellular Ca<sup>2+</sup>mobilizing activity, J. Biol. Chem. 264 (1989) 1608–1615.
- [29] C. Schultz, Prodrugs of biologically active phosphate esters, Bioorg. Med. Chem. 11 (2003) 885–898.
- [30] T. Posternak, E.W. Sutherland, W.F. Henion, Derivatives of cyclic 3',5'adenosine monophosphate, Biochim. Biophys. Acta 65 (1962) 558–560.
- [31] R.Y. Tsien, A non-disruptive technique for loading calcium buffers and indicators into cells, Nature 290 (1981) 527–528.
- [32] A.B. Jansen, T.J. Russell, Some novel penicillin derivatives, J. Chem. Soc. 65 (1965) 2127–2132.
- [33] R.Y. Tsien, Fluorescent probes of cell signaling, Annu. Rev. Neurosci. 12 (1989) 227–253.
- [34] W. Li, C. Schultz, J. Llopis, R.Y. Tsien, Membrane-permeant esters of inositol polyphosphates, chemical syntheses and biological applications, Tetrahedron 53 (1997) 12017–12040.
- [35] L. Mackenzie, H.L. Roderick, A. Proven, S.J. Conway, M.D. Bootman, Inositol 1,4,5-trisphosphate receptors in the heart, Biol. Res. 37 (2004) 553–557.
- [36] S.J. Conway, G.J. Miller, Biology-enabling inositol phosphates, phosphatidylinositol phosphates and derivatives, Nat. Prod. Rep. 24 (2007) 687–707.
- [37] R. Parkesh, A.M. Lewis, P.K. Aley, et al., Cell-permeant NAADP: a novel chemical tool enabling the study of Ca<sup>2+</sup> signalling in intact cells, Cell Calcium 43 (2008) 531–538.
- [38] J.T. Axelson, J.W. Bodley, T.F. Walseth, A volatile liquid chromatography system for nucleotides, Anal. Biochem. 116 (1981) 357–360.
- [39] H.C. Lee, R. Aarhus, ADP-ribosyl cyclase: an enzyme that cyclizes NAD<sup>+</sup> into a calcium-mobilizing metabolite, Cell. Regul. 2 (1991) 203–209.
- [40] H.C. Lee, C. Munshi, R. Graeff, Structures and activities of cyclic ADP-ribose, NAADP and their metabolic enzymes, Mol. Cell. Biochem. 193 (1999) 89–98.
- [41] C. Munshi, D.J. Thiel, I.I. Mathews, R. Aarhus, T.F. Walseth, H.C. Lee, Characterization of the active site of ADP-ribosyl cyclase, J. Biol. Chem. 274 (1999) 30770–30777.
- [42] C. Schultz, M. Vajanaphanich, H.G. Genieser, B. Jastorff, K.E. Barrett, R.Y. Tsien, Membrane-permeant derivatives of cyclic AMP optimized for high potency, prolonged activity, or rapid reversibility, Mol. Pharmacol. 46 (1994) 702–708.
- [43] H.C. Lee, R. Aarhus, A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose, J. Biol. Chem. 270 (1995) 2152–2157.
- [44] M. Whitaker, Calcium and mitosis, Prog. Cell Cycle Res. 3 (1997) 261-269.
- [45] S.R. Vasudevan, A.M. Lewis, J.W. Chan, et al., The calcium-mobilizing messenger nicotinic acid adenine dinucleotide phosphate participates in sperm activation by mediating the acrosome reaction, J. Biol. Chem. 285 (2010) 18262–18269.
- [46] A.H. Guse, Second messenger function and the structure-activity relationship of cyclic adenosine diphosphoribose (cADPR), FEBS J. 272 (2005) 4590–4597.
- [47] B.V.L. Potter, T.F. Walseth, Medicinal chemistry and pharmacology of cyclic ADP-ribose, Curr. Mol. Med. 4 (2004) 303–311.
- [48] E. Clementi, M. Riccio, C. Sciorati, G. Nisticò, J. Meldolesi, The type 2 ryanodine receptor of neurosecretory PC12 cells is activated by cyclic ADP-ribose. Role of the nitric oxide/cGMP pathway, J. Biol. Chem. 271 (1996) 17739–17745.

- [49] J. Yue, W. Wei, C.M.C. Lam, et al., CD38/cADPR/Ca<sup>2+</sup> pathway promotes cell proliferation and delays nerve growth factor-induced differentiation in PC12 cells, J. Biol. Chem. 284 (2009) 29335–29342.
- [50] L.A. Greene, A.S. Tischler, Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor, Proc. Natl. Acad. Sci. U S A 73 (1976) 2424–2428.
- [51] D. Zacchetti, E. Clementi, C. Fasolato, et al., Intracellular Ca<sup>2+</sup> pools in PC12 cells. A unique, rapidly exchanging pool is sensitive to both inositol 1,4,5trisphosphate and caffeine-ryanodine, J. Biol. Chem. 266 (1991) 20152–20158.
- [52] M. Endo, Calcium-induced calcium release in skeletal muscle, Physiol. Rev. 89 (2009) 1153–1176.
- [53] M. Kolisek, A. Beck, A. Fleig, R. Penner, Cyclic ADP-ribose and hydrogen peroxide synergize with ADP-ribose in the activation of TRPM2 channels, Mol. Cell. 18 (2005) 61–69.
- [54] O. Thastrup, A.P. Dawson, O. Scharff, et al., Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage, Agents Actions 27 (1989) 17–23.
- [55] J. Gafni, J.A. Munsch, T.H. Lam, et al., Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor, Neuron 19 (1997) 723–733.