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8-pCPT-2′-O-Me-cAMP-AM: An Improved Epac-Selective cAMP Analogue

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Cyclic adenosine monophosphate (cAMP) is a common second messenger involved in the regulation of many different cellular processes through the activation of protein kinase A (PKA), exchange protein directly activated by cAMP (Epac) and cyclic-nucleotide-regulated ion channels.[1] Adenylyl cyclases are responsible for catalysing the formation of cAMP from ATP. Levels of cAMP can be raised in cells in response to a large variety of extracellular stimuli, which act via receptors coupled to heterotrimeric G proteins, which stimulate the activity of adenylyl cyclase. In addition, cAMP levels are controlled by phosphodiesterases (PDE), which catalyse the degradation of cAMP to AMP. In cells, cAMP levels can be artificially elevated by forskolin, which activates adenylyl cyclase directly. Furthermore, cAMP levels can be raised by inhibiting PDEs. These approaches are commonly used in tissue culture experiments, but, by generating cAMP, they do not discriminate between the various target proteins that are activated. Alternatively, membrane-permeable cAMP analogues, which selectively interact with particular receptor proteins, can be applied. For example, signalling pathways activated by Epac and PKA can be distinguished by using 8-pCPT-2′-O-Me-cAMP and 6-Bnz-cAMP, respectively.[2]

Epac is a guanine nucleotide exchange factor for the small G protein Rap. Rap cycles between a signalling-inactive GDP-bound state and a signalling-active GTP-bound state. cAMP-activated Epac catalyses the exchange of Rap-bound GDP for GTP. Epac and Rap function in a number of different cellular processes including insulin secretion, inhibition of cell scattering, neurotransmitter release and cAMP-induced barrier function in endothelial cells.[3]

Even though 8-pCPT-2′-O-Me-cAMP has become a widely used tool in Epac-related research, its biological application is limited by its low membrane permeability, caused by the negatively charged phosphate. However, the negatively charged singly bonded oxygen on the phosphate group can be masked by labile esters. Such a precursor is expected to enter the cell efficiently, where the ester is hydrolysed either directly by water or by cellular esterases to liberate the active compound.[4]

We therefore synthesised 8-pCPT-2′-O-Me-cAMP-AM from 8-pCPT-2′-O-Me-cAMP, whereby acetoxymethyl bromide was used as a donor for the AM group. The product that was obtained had a purity exceeding 97% and consisted of a mixture of the equatorial and the axial isomers of the ester (Figure S1 in the Supporting Information, Scheme 1). Even though the isomers could be resolved by repetitive analytical HPLC runs, efficient separation on a preparative scale was not possible. Orange peel acetylesterase and esterase from porcine liver cleaved the equatorial isomer about five times more efficiently than the axial isomer within minutes (data not shown). The pharmacokinetics of both isomers are thus expected to be similar, justifying the application of a mixture of both isomers to cells. In any case, the isomeric ratio of an individual synthesis can be easily quality controlled by ^{31}P NMR (Figure S1).

To compare the efficiency of 8-pCPT-2′-O-Me-cAMP-AM and 8-pCPT-2′-O-Me-cAMP in activating Epac1 in vivo, an Epac1-based fluorescence resonance energy transfer (FRET) probe was used. In this assay, activation of Epac1 by the binding of cAMP to the Epac1-FRET probe is measured as a reduction in the FRET signal.[5] A431 cells transfected with the FRET probe were stimulated with 8-pCPT-2′-O-Me-cAMP-AM or 8-pCPT-2′-O-Me-cAMP (Figure 1). Stimulation of cells with 100 μM 8-pCPT-2′-O-Me-cAMP resulted in a decrease of the FRET signal that was approximately one order of magnitude slower than the decrease obtained upon stimulation with 1 μM 8-pCPT-2′-O-Me-cAMP-AM. Furthermore, activation of Epac1 following stimulation with 100 μM 8-pCPT-2′-O-Me-cAMP could be further enhanced by the addition of forskolin, whereas 1 μM 8-pCPT-2′-O-Me-cAMP-AM induced maximal activity of Epac1 under the given conditions. The activation of Epac by 8-pCPT-2′-O-Me-cAMP-AM occurs within one minute after application. This is comparable with the kinetics of forskolin-induced Epac activation, and thus 8-pCPT-2′-O-Me-cAMP-AM mimics the "natural" response time of the signalling pathway.

The activity of endogenous Epac can be monitored by isolating selectively Rap·GTP from cell lysates. Primary human umbilical vein endothelial cells (HUVEC) were stimulated with different concentrations of 8-pCPT-2′-O-Me-cAMP and 8-pCPT-2′-O-Me-cAMP-AM (Figure 2A). Partial activation of Rap was induced by 10 μM 8-pCPT-2′-O-Me-cAMP, and full activation of the G protein was stimulated by 100 μM 8-pCPT-2′-O-Me-cAMP. In contrast, treatment of the cells with just 0.1 μM 8-pCPT-2′-O-Me-cAMP-AM was sufficient to induce full Rap activation.

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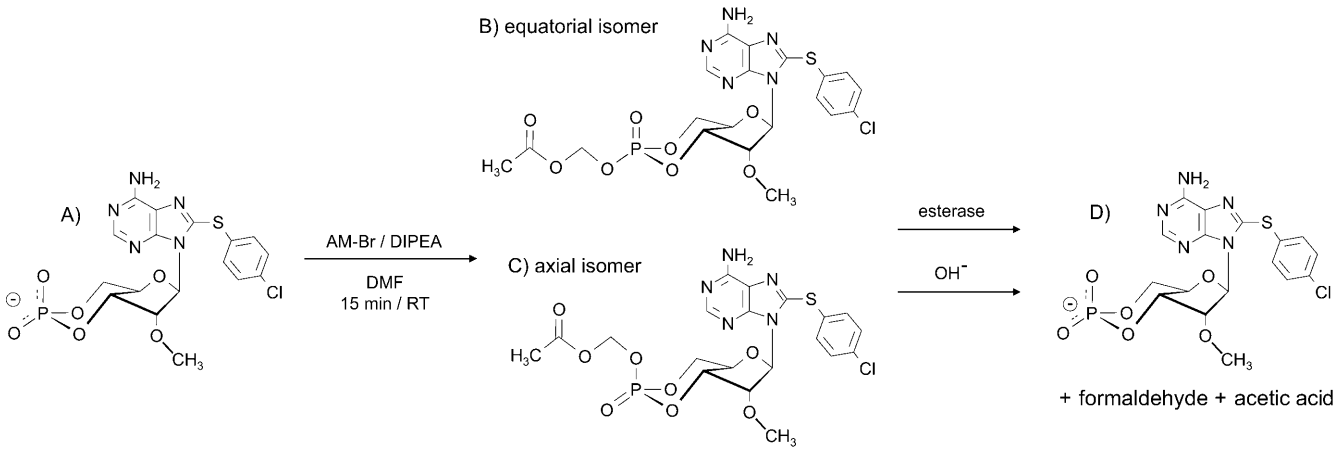

Scheme 1. Synthesis and application of 8-pCPT-2′-O-Me-cAMP-AM. A) 8-pCPT-2′-O-Me-cAMP is converted into 8-pCPT-2′-O-Me-cAMP-AM with acetoxymethyl bromide as a donor of the acetoxymethyl group in the presence of *N*,*N*-diisopropylethylamine in *N*,*N*-dimethylformamide. A mixture of the equatorial (B) and axial (C) isomers of the ester is obtained. D) 8-pCPT-2′-O-Me-cAMP-AM is cleaved by esterases to 8-pCPT-2′-O-Me-cAMP and the by-products formaldehyde and acetic acid.

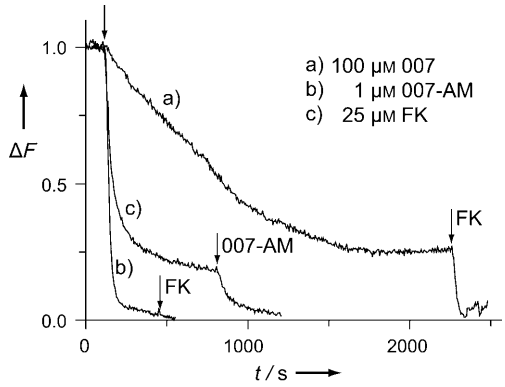

Figure 1. Epac1 activation in A431 cells. A431 cells were transfected with the Epac1-FRET probe and stimulated with 100 μM 8-pCPT-2′-O-Me-cAMP (007) or 1 μM 8-pCPT-2′-O-Me-cAMP-AM (007-AM) followed by 25 μM forskolin (FK) or stimulated by 25 μM forskolin followed by 1 μM 8-pCPT-2′-O-Me-cAMP-AM. The FRET signal was monitored over time and plotted as normalised change in FRET (ΔF); the traces are representatives of ten independent experiments.

To determine if 8-pCPT-2′-O-Me-cAMP-AM could efficiently stimulate Rap-dependent processes, biological assays were carried out. In HUVECs, Rap induces a tightening of cell–cell junctions that can be measured as an increase in the electrical resistance of a cell layer grown on an electrode. 8-pCPT-2′-O-Me-cAMP-AM induced junction tightening at much lower concentrations than 8-pCPT-2′-O-Me-cAMP (Figure 2B). Similarly, 8-pCPT-2′-O-Me-cAMP-AM induced adhesion of Jurkat-Epac1 cells to fibronectin more efficiently than 8-pCPT-2′-O-Me-cAMP (Figure 2C).

Thus, 8-pCPT-2′-O-Me-cAMP-AM induces Epac1 and Rap1 activation at concentrations that are two to three orders of magnitudes lower than those required of the parent compound. In HUVECs, sustained Rap1 activation was observed after application of only 0.01 μM 8-pCPT-2′-O-Me-cAMP-AM (Figure 2A). This concentration is far below the AC_{50} of 8-pCPT-2′-O-Me-cAMP for Epac1, which was determined to be 1.8 μM in vitro.[6] This indicates that 8-pCPT-2′-O-Me-cAMP accumulates in the cell after the cleavage reaction, which is in accordance with results from other cyclic nucleotide AM esters.[7] Indeed, 8-pCPT-2′-O-Me-cAMP-AM seems to enter cells much more quickly than 8-pCPT-2′-O-Me-cAMP, as shown by the more rapid activation of the Epac1-FRET probe by 8-pCPT-2′-O-Me-cAMP-AM in comparison to 8-pCPT-2′-O-Me-cAMP (Figure 1).

The biological selectivity of 8-pCPT-2′-O-Me-cAMP is probably its greatest benefit for biological research. However, the application of 8-pCPT-2′-O-Me-cAMP-AM causes an accumulation of 8-pCPT-2′-O-Me-cAMP in cells. To exclude the possibility that a putative high intracellular concentration of 8-pCPT-2′-O-Me-cAMP caused side effects, 8-pCPT-2′-O-Me-cAMP-AM was applied to cells expressing a PKA-based FRET probe (Figure 3A). Upon application of 1 μM 8-pCPT-2′-O-Me-cAMP-AM, no change in the PKA-FRET signal was observed. In addition, the phosphorylation status of a PKA substrate, vasodilator-stimulated phosphoprotein (VASP), was monitored as a biological measure of the activity of the enzyme. Whereas a clear band shift of VASP is observed after stimulation with forskolin, no effect is observed with 1 μM 8-pCPT-2′-O-Me-cAMP-AM (Figure 3B).

To summarise, we have described the synthesis of 8-pCPT-2′-O-Me-cAMP-AM, a precursor that selectively activates Epac and is more efficiently delivered into cells than its parent compound. 8-pCPT-2′-O-Me-cAMP-AM works with high efficiency under biological conditions by stimulating Epac and by activating Rap1-dependent processes, as demonstrated in two model systems. We found that 8-pCPT-2′-O-Me-cAMP-AM is stable for at least two hours in aqueous solution, but in general less stable in sera containing esterases. In addition, it is possible that toxic side effects might be caused by the by-products of the esterase reaction. However, related prodrugs, such as pivampicillin or Hepsera™, both of which release a carboxylic acid and formaldehyde, or Enalapril™ or acetylsalicylic acid, both of which release acetic acid, are in clinical use arguing for the general safety of AM-ester-based precursors.[8] 8-pCPT-2′-O-Me-

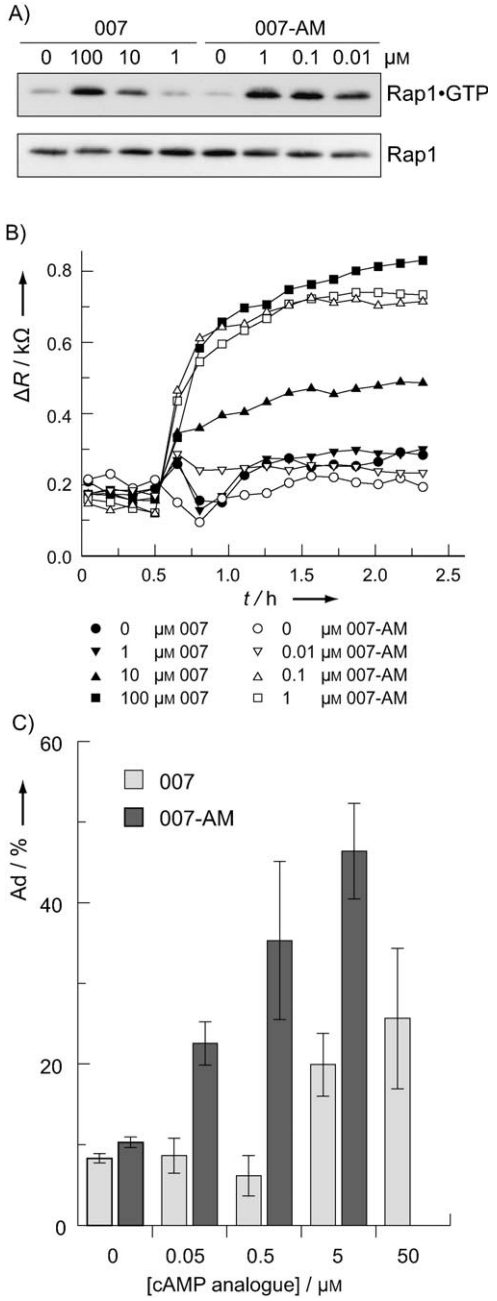

Figure 2. 8-pCPT-2′-O-Me-cAMP-AM acts efficiently on cells. A) HUVECs were stimulated with different concentrations of 8-pCPT-2′-O-Me-cAMP (007) or 8-pCPT-2′-O-Me-cAMP-AM (007-AM), and the levels of Rap1•GTP were measured. B) HUVECs grown on an electrode were stimulated with different concentrations of 8-pCPT-2′-O-Me-cAMP or 8-pCPT-2′-O-Me-cAMP-AM as indicated. The change in transendothelial electrical resistance (ΔR) was measured in real time. C) Jurkat-Epac1 cells were stimulated with different concentrations of 8-pCPT-2′-O-Me-cAMP or 8-pCPT-2′-O-Me-cAMP-AM and seeded on fibronectin coated plates. Adhesion (Ad) was measured after 15 min.

cAMP-AM is thus expected to become a powerful tool in Epac- and PKA-related research.

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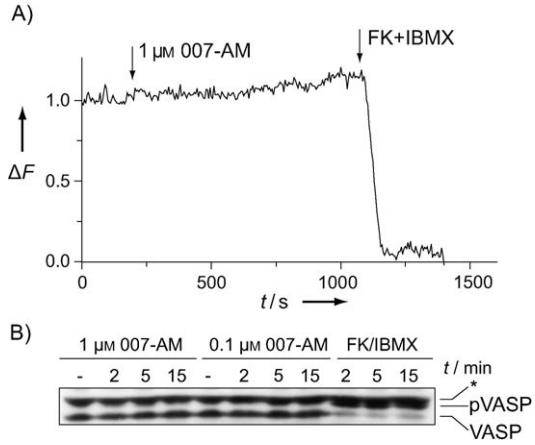

Figure 3. Selectivity of 8-pCPT-2′-O-Me-cAMP-AM. A) Ovcar3 cells were transfected with the PKA-FRET probe and stimulated successively with 1 μM 8-pCPT-2′-O-Me-cAMP-AM and a combination of 25 μM forskolin (FK) and 100 μM 3-isobutyl-1-methylxanthine (IBMX). The FRET signal was monitored over time and is plotted here as normalised change in FRET (ΔF). The trace is a representative of three independent experiments. B) Ovcar3 cells were stimulated with 1 μM or 0.1 μM of 8-pCPT-2′-O-Me-cAMP-AM or with a combination of 50 μM forskolin and 500 μM IBMX (and PDE inhibitor), and phosphorylation of VASP was monitored by a phosphorylation-induced mobility shift after the indicated time points. The band marked by asterisk corresponds to unspecific background staining.

Keywords: cAMP · Epac · esters · precursors · selectivity · synthetic drugs

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