Biochemical and Biophysical Research Communications 439 (2013) 137–141

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

A derivative of ascorbic acid modulates cAMP production

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ARTICLE INFO

Article history:
Received 27 July 2013
Available online 11 August 2013

Keywords:
Ascorbic acid
Cyclic AMP
Antioxidants

ABSTRACT

We reported, in previous experiments, that AA is a global regulator of cAMP pools. In this study, we demonstrate that K873, an analog of AA we synthesized and presenting antiproliferative properties, has also an impact on cAMP production. However, K873 has no antioxidant activity, at the contrary of AA. It definitively demonstrates that action of AA on the cAMP production is not linked to antioxidant activity. These data suggest that AA, and derivatives of this molecule, could be promising drug acting on biological processes that are under the control of cAMP dependent pathway.

1. Introduction

In 2004, we demonstrated that treatment of a mouse model of the Charcot-Marie-Tooth 1A disease (an inherited peripheral neuropathy) by high doses of ascorbic acid (AA) reverts at least partly the phenotype of transgenic mice [1]. This disease is due to the over expression of a major gene of myelin, *PMP22*, and AA treatment results in lowering *PMP22* expression. In further experiments we demonstrated that Schwann cell lines incubated with increasing concentrations of AA results in a dosage dependant inhibition of *PMP22* expression. In addition, an evaluation of the intracellular cAMP pool demonstrated that this pool is decreased when cells are incubated with increasing concentration of AA [2]. This inhibition is specific to AA and is not shared by other antioxidants [3]. Recently, using classical enzymatic experiments, we demonstrated that AA is a competitive inhibitor of adenylate cyclase, probably because AA and ATP present both the furanic ring of ribose [4]. Therefore, AA is probably a molecule that inhibits expression of genes under the control of cAMP dependant pathway [for review, 5]. Finally, we demonstrated that AA inhibits cell proliferation *in vitro* as well as *in vivo* [6]. These studies led us to propose AA as a potential player in cell differentiation [5].

In order to validate our previous work and to develop potentially more active molecules, we decided to synthesize derivatives of ascorbic acid presenting a structure more similar to ATP, by adding phosphate or adenine lateral chains to the furanic ring. We synthesize a small series of derivatives of AA and tested their antiproliferative properties [7]. One of these new molecule (K873, structure and synthesize has already been described in [7]), presenting *in vitro* and *in vivo* antiproliferative and cytotoxic agent on human cancer cells, has been selected.

In this article we demonstrated that this molecule lowers production of cAMP as ascorbic acid does and that it inhibits *PMP22* expression, as AA does but a lower concentration. We also demonstrated that other vitamins with antioxidant properties, did not act on the cAMP pool, at the exception of β-carotene that increases the intracellular cAMP pool. The potential impact of these findings on cellular processes, as cell proliferation, will be discussed.

2. Materials and methods

2.1. Sciatic nerves organ-culture

Sciatic nerves of CMT mice (C22) were surgically extracted and deposited in 12 wells microplate containing a specific medium composed of RPMI 1640 with 25 mM HEPES supplemented with 15% fetal bovine serum and 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO®). Microplates were incubated at 37 °C in 5% CO_2/95% air.

2.2. Schwann cell line culture

Cells were purchased from the American Type Culture Collection (ATCC). S16 line was derived from a primary culture of rat sciatic nerve Schwann cells (*Rattus norvegicus*). Cells were grown according to the manufacturer's instructions in RPMI 1640 with 25 mM HEPES supplemented with 10% fetal bovine serum (GIBCO®) at 37 °C in 5% CO_2/95% air. Cells were counted under a

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http://dx.doi.org/10.1016/j.bbrc.2013.08.004

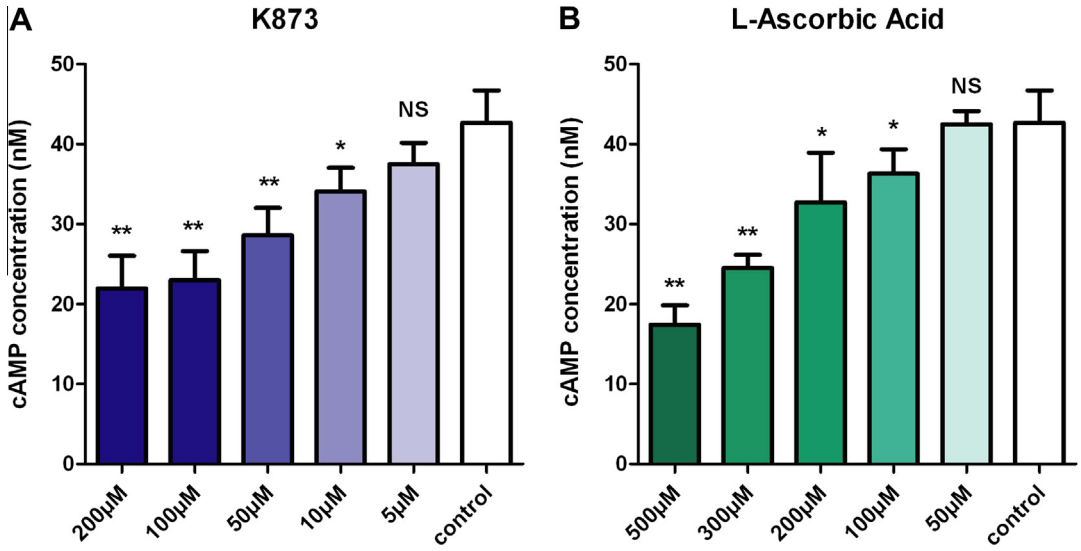


Fig. 1. Schwann cell lines have been incubated (see methods) either with increasing doses of K873 (A) or AA (B). Production of cAMP has been evaluated as described in methods.

microscope (10× lens) using a Neubauer hemocytometer. Cell viability was estimated using Trypan Blue staining.

2.3. RNA extraction and RT-PCR

Total RNA was extracted using TRIZOL® reagent (Invitrogen™) following the manufacturer's instructions. To double-check RNA integrity prior to use, the quality and concentration of each sample was tested on an Agilent 2100 Bioanalyzer.

2 μg of total RNA from each sample (nerves) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen™) to produce cDNA. To compare the levels of *PMP22* mRNA in nerves treated by Ascorbic Acid, K873 or without treatment conditions, cDNA was amplified with a thermocycler (Eppendorf-Mastercycler®) using two primers pairs. The first one, primers was specific for exon1A of *PMP22* gene, the variant only expressed in Schwann cells. The second one, for normalization, primers was specific for mouse β-actin gene. The PCR product crosses an intron and does not amplify from genomic DNA. Amplification was for 30 cycles. After, PCR products were analyzed on 2% agarose gels. Pictures were captured using a digital imager. Evaluation of band intensity was accomplished using the ImageJ package.

2.4. RNA extraction and qRT-PCR

Total RNA of nerves was extracted from cells using TRIZOL® reagent (Invitrogen™) following the manufacturer's instructions. To double-check RNA integrity prior to use, the quality and concentration of each sample was tested on an Agilent 2100 Bioanalyzer.

Quantitative reverse-transcriptase PCR was performed using a LightCycler® 480 Real-Time PCR System (Roche) with a universal probe library (UPL). 2 μg of total RNA from each sample (nerves) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen™) to produce cDNA. Mouse β-actin was used for data normalization. The results were processed using comparative CT, where the amount of the target, normalized to the endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta CT}$.

2.5. Evaluation of cAMP concentration

Intracellular cAMP level was measured using cAMP-Glo™ Assay (Promega Corp.). Briefly, 100 μl of S16 cells solution (5×10^4cells/ml) was deposited in a sterile 96-wells microplate (culture treated) and incubated at 37 °C in 5% CO_2/95% air. After cell adhesion (24 h), culture medium was replaced by 20 μl of our compounds solutions, prepared in "induction buffer". Each solution dilution was tested in triplicate, and wells containing S16 cells in 20 μl of induction buffer alone were used as controls like basal level of intracellular cAMP. Then, assay was performed according to the manufacturer's instructions. At the end, plates were read using a microplate reader (Victor™ X4, PerkinElmer®). According to kit instructions, "Induction Buffer" was composed with PBS containing 500 μM of 3-isobutyl-1-methylxanthine (IBMX) and 100 μM of 4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one (Ro20-1724). The IBMX and Ro20-1724 are inhibitors of phosphodiesterases and used to prevent cAMP hydrolysis during the assay. In summary, this assay evaluates cAMP production, as a consequence of adenylate cyclase activity.

2.6. Evaluation of antioxidant potential by ORAC technology

Oxygen Radical Absorbance Capacity was measured according to manufacturer kit instructions (YELEN, France). The principle is to register degradation kinetics of a fluorescent probe subjected to oxidative stress. Briefly, 25 μl of compounds solutions were diluted in 150 μl of fluorescein solution and incubated 10 min at 37 °C. Then, 25 μl of AAPH (peroxyl radicals' generator) solution (37 °C) was added and fluorescence (Ex485/Em530) kinetics was recorded with a fluorimeter microplate reader (Infinite® M200, TECAN). Trolox was used as reference of antioxidant molecules.

2.7. Statistical analysis

Statistical analysis was performed using Prism® v5.0 GraphPad software. For qPCR analysis and cAMP-Glo assay, we used the Mann-Whitney two-tailed statistical significance test, with a confidence interval of 95%. For ORAC assay, we used the "One-way ANOVA" analysis with "Bonferroni's Multiple Comparison Test". A p-value lower than 0.05 was considered significant. One asterisk (*) indicates a p-value <0.05, two asterisks (**) a p-value <0.01, and three asterisks (***) a p-value <0.001.

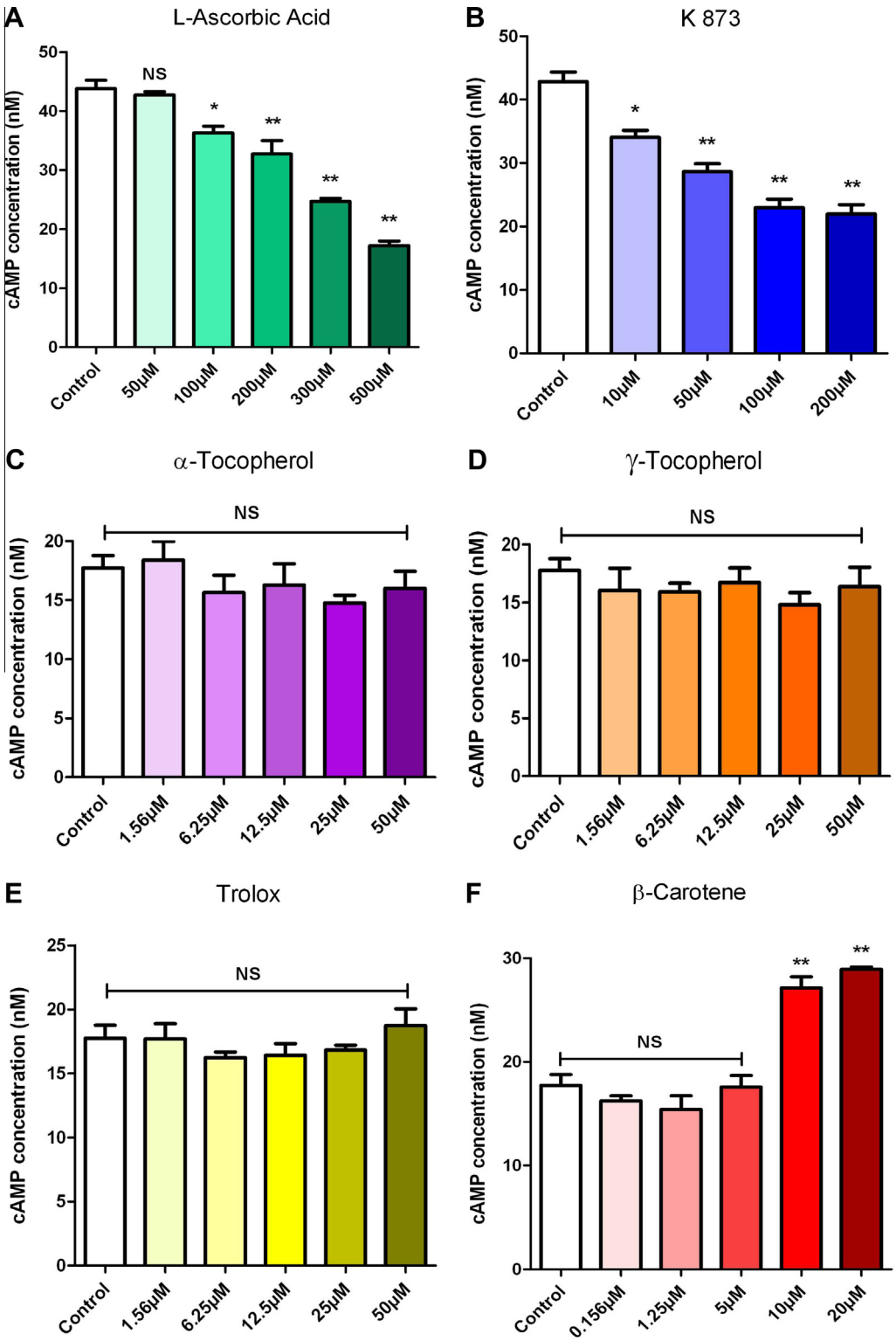


Fig. 2. The same experiment has been conducted with AA, K873, Trolox, α- and γ-Tocopherol and β-carotene. cAMP pool has been evaluated as in Fig. 2.

3. Results and discussion

3.1. Action of AA and KN873 on cAMP productionl

We previously demonstrated that AA is a global regulator of cAMP pool [4]. In this article we asked the same question for a derivative of AA, K873. Is this molecule, as AA, acts on cAMP production? As our previous work has been performed using Schwann cell lines, we used the same line in the present experiments (S16, [8]). cAMP production by S16 cells treated or not with AA or K873, was evaluated (see methods). We could observe in Fig. 1 that incubation of cells with increasing concentration of either AA or K873 lowers cAMP production. This effect is dosage dependent and IC_{50} is about 400 μM for AA and 80 μM for K873.

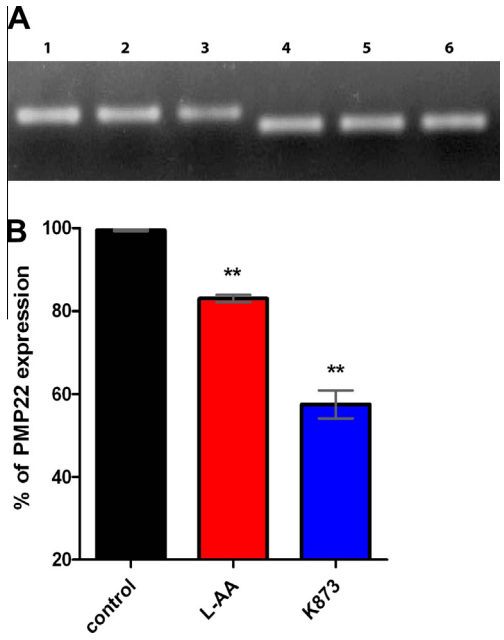
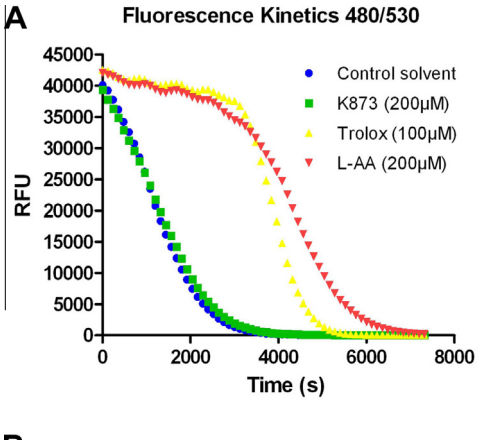


Fig. 3. Sciatic nerves have been incubated in a specific medium (see methods) without addition (1 and 4), with 0.5 mM of AA (2 and 5) and with 0.08 mM of K873 (3 and 6) during 24 h. RNAs was extracted. (A) RT-PCR has been performed using primers specific of *PMP22* Schwann specific transcript (1–3) or β actin (4–6) as reference. (B) Same as in A but using qPCR technology (see methods).



B

Bonferroni's Multiple Comparison Test	ORAC value	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Control solvent vs K873 (200M)	**Control = 0.02**	-231.4	0.08609	No	ns	-7381 to 6918
Control solvent vs Trolox (100M)	**Trolox = 1**	-13900	5.17	Yes	***	-21050 to -6747
Control solvent vs L-AA (200M)		-15800	5.878	Yes	***	-22950 to -8650
K873 (200M) vs Trolox (100M)	**K873 = 0.02**	-13670	5.084	Yes	***	-20820 to -6516
K873 (200M) vs L-AA (200M)		-15570	5.792	Yes	***	-22720 to -8418
Trolox (100M) vs L-AA (200M)	**L-AA = 1**	-1902	0.7077	No	ns	-9052 to 5248

Fig. 4. (A) Antioxidant activity of K873 has been evaluated using the ORAC technology. Antioxidant activity of AA and Trolox was also conducted as references. (B) ORAC index of K873, AA and trolox and statistical analysis of results of ORAC assays.

In a further experiment, we compared the impact of other vitamins (A, D, E) or molecule (Trolox), described as antioxidant, on cAMP production (Fig. 2). We could see that no molecules have an effect on the cAMP production at standard concentrations, except β-carotene. However, this molecule did not lower cAMP production but increases it. This interesting data will be discussed below. However, as a conclusion, K873 acts on cAMP production as AA does.

3.2. Action on PMP22 expression

Our preceding work on CMT1A disease demonstrated that AA could act on gene expression via the modulation of intracellular cAMP [1]. We demonstrated that AA inhibits expression of *PMP22*, the gene involved in CMT1A, in a dosage sensitive way [2]. Therefore, we evaluated the action of K873 on expression of this gene. In order to be as close as possible of *in vivo* situation, we developed a medium able to maintain surgical pieces of sciatic nerve alive, without differentiation, during 1/2 days (see methods). AA or K873 were added to the medium, RNAs extracted and expression of the Schwann specific transcript of *PMP22* was analyzed by RT-PCR and qPCR. We could observe in Fig. 3 that K873 inhibits *PMP22* expression at lower doses than AA. This observation suggests that this molecule is able to act on the expression of the same genes as AA. Moreover, K873 could be a potential new drug for CMT1A, inhibiting *PMP22* over expression. Finally, this molecule has been found not toxic in *in vitro* cellular tests [7].

3.3. KN873 is not an antioxidant

We demonstrated, in previous experiments, that AA lowers, in a dose dependent, the intracellular cAMP pool. We also demonstrated that this activity is not shared by other vitamins presenting antioxidant properties. However, AA is an antioxidant. We thus tested a potential antioxidant activity of K873, using the ORAC technology, using AA and trolox as references [9–11]. Results are presented in Fig. 4. These results demonstrated that K873 has no significant antioxidant property. This demonstrates that action of K873 on cAMP pool is not due to an antioxidant property. Moreover it confirms that the impact of AA on cAMP production is thus not due to its antioxidant properties.

4. Conclusion

We demonstrated in previous work, that ascorbic acid is an inhibitor of the expression of the gene *PMP22* [1], a gene over expressed in CMT1A. We demonstrated, in further works, that this inhibition is due to the action of AA on the intracellular pool of cAMP [2–4]. In this article, we demonstrate that K873, an analog of AA, is also a dose dependent inhibitor of cAMP production, as AA is (although K873 seems to act at lower concentration). Moreover, we demonstrated that K873 inhibits expression of *PMP22*, the gene involved in CMT1A and under the control of cAMP dependent pathways.

AA has been described for a long time only as an antioxidant, and its biological activity linked to this property [5]. However, there is actually an increasing number of data that demonstrate that AA has function that could not be linked to its antioxidant properties [12–14]. In this study, we demonstrate that an analog of AA, K873, has no significant antioxidant properties but this molecule acts in cAMP production, and gene expression, as AA did. It definitively demonstrates that action of AA on the cAMP pool is not linked to antioxidant activity.

These data suggest that AA, and derivatives of this molecule, could be a promising drugs acting on biological processes that are under the control of cAMP dependent pathway. As we already observed, these molecules are weak inhibitors and are thus probably global regulator of cAMP pool. This is probably why they are not toxic [4]. It is well known that AA is not toxic, even at high concentrations, but we recently demonstrated that K873 is also not toxic using *in vitro* cellular tests [7].

As a last conclusion, we tested the principle vitamin precursor (A, C–E), that has been associated with an antioxident activity. We detected only one, β-carotene, the precursor of retinoic acid, excepted AA, that acts on cAMP production. However, this molecule

did not inhibit cAMP production but enhances it. This is particularly interesting as retinoic acid has been demonstrated as an activator of cAMP production [15]. On the contrary AA, and its analogs, lower cAMP pool and repress cell proliferation. These two molecules could thus act in competition on different cell processes like cell differentiation. This will be a very promising way in future researches.

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