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Ascorbic acid is a regulator of the intracellular cAMP concentration: Old molecule, new functions?

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Abstract Recently, using an animal model of Charcot-Marie-Tooth human disorder, we showed that ascorbic acid (AA) represses *PMP22* gene expression by acting on intracellular cAMP concentrations. In this work, we present kinetics data on the inhibitory effect of AA upon adenylate cyclase activity. The data show that this molecule acts as a competitive inhibitor of the enzyme, a finding that opens new pharmacological avenues.

Keywords: Ascorbic acid; Adenylate cyclase; Gene expression; CMT disorder

1. Introduction

Using a YAC transgenic murine line (C22) that mimics the human Charcot-Marie-Tooth type 1A disorder (CMT-1A) [1], we showed that high concentrations of ascorbic acid (AA) can result in a significant amelioration of the CMT phenotype in mice [2]. AA treatment reduced *PMP22* expression to a level below which that is necessary to correct the CMT-1A phenotype [2]. These data provided the background for a clinical phase II/III trial in humans.

Then, we showed that AA inhibits cAMP-dependent expression of the *PMP22* gene, and that this action was dose dependent [3]. According to this work high doses of AA added to the Schwann cells culture resulted in decrease of the intracellular concentration of the second messenger, and we proposed that AA might be considered as a "global regulator" of intracellular cAMP level [3]. By this property AA would affect any pathway in which cAMP is implicated. In addition, we demonstrate that this property is not shared with other anti oxidants [3].

Intracellular cAMP concentrations are controlled by two mechanisms: a production pathway of cAMP via the activity of nine adenylate cyclase isoforms and a degradation pathway via the activity of specific phosphodiesterases. Both pathways could be modulated either by variations of gene expression encoding the corresponding enzymes or by a direct modification of their enzymatic activities. In the present work, we address this question using two independent set of experiments:

1. We analyzed first the expression of genes coding for adenylate cyclase, and demonstrate that AA did not affect their expression.
2. The second hypothesis is that AA directly affects adenylate cyclase activity. To this end we have analyzed in vitro kinetics data of the action of AA on a membrane mixture isolated from murine Schwann cell cultures. This allowed us evaluating the inhibitory mode of action of AA on adenylate cyclase activity.

2. Materials and methods

2.1. Murine Schwann cell culture

The MSC80 cell line used in this study was derived from murine sciatic nerve primary Schwann cells [4]. These cells express the same set of markers than those expressed in vivo by Schwann cells. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), as described previously [4]. These cells are bipolar and their doubling time was approximately 17 h.

2.2. RNA extraction and RT-PCR

Total RNA was extracted from cell cultures using the Trizol reagent (Invitrogen) and resuspended in RNAase-free water. The concentration of total RNA was determined using the NanoDrop technology instrument. Two micrograms total RNA was then used for RT reaction, which was performed using SuperScript II kit (Invitrogen) according to the manufacturer's instructions. The resulted cDNAs were stored at −80 °C until use.

Levels of mRNA encoding adenylate cyclase isoforms were quantified by real-time RT-PCR employing the LightCycler 480 (Roche). All measurements were performed in triplicate in SyberGreen Master Mix (Roche) using 96-well optical PCR plates.

Appropriate primers for the adenylate cyclase isoforms were designed using the primer3 web site (http://www-genome-wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Ribosomal 18S RNA was used as an internal standard.

For each reaction, 5 μl diluted cDNA (1/50), 12.5 μl SyberGreen Master Mix, 0.5 μl reverse primer (1 μM), 0.5 μl forward primer (10 μM) and 6.5 μl sterile water were employed.

Real-time PCR was carried out according to a standard manufacturer's protocol involving 40 cycles of denaturation–annealing. Expression level of each gene were analysed by the relative quantification calculation software of the Roche light cycler.

2.3. Adenylate cyclase assay

Cultured MSC80 cells were homogenized in 2 ml TEOP buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM orthovanadate, and protease inhibitors (1 mM PMSF and 1/2 tablet of complete mini peptidase inhibitors per 50 ml buffer; Roche Applied Science). Unbroken cells and nuclei were removed by low-speed centrifugation at 900 × *g* for 5 min at 4 °C. Then the supernatant was centrifuged at 19,000 × *g* for 20 min at 4 °C and the resulting pellet was

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resuspended in 200 μl buffer containing 20 mM Tris–HCl (pH 7.5) and 1 mM EDTA. These membrane preparations after appropriate dilutions (2 μg protein per μl buffer composed of 20 mM Tris–HCl, 1 mM EDTA and 1 mM DTT) were used consequently for adenylate cyclase activity assays.

Assay for the determination of adenylate cyclase activity was based on the estimation of the quantity of cAMP formed under standard conditions (linearity versus reaction time and versus enzyme concentration) using a highly specific quantitative test (DE0355; R&D Systems). This assay is based on the competitive binding technique in which cAMP present in a sample competes with a fixed amount of Horseradish (HPR)-labeled cAMP for sites on a mouse monoclonal antibody. During the incubation, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determined the bound enzyme activity. The color development is stopped and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of cAMP in the sample. The reaction mixture (total volume of 0.1 ml) contained 25 μg total membrane proteins, 50 mM triethanolamine–HCl (pH 7.4), 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 1 mM isobutyl-methylxanthine, 0.2% bovine serum albumin (BSA), and 50 μM GTP. After incubation for 10 min at 30 °C, 5 μl of substrate solution (1 mM ATP) were added to start the reaction, which was terminated 5 min later by adding 0.4 ml of 0.1 M HCl. A standard curve with known concentrations of cAMP was used to express data in pmol cAMP per min. Protein content of membrane preparations was estimated using the bicinchoninic acid (BCA)-protein assay (Sigma–Aldrich).

Inhibition studies were performed using fixed concentrations of neutralized Na-ascorbate preparations for different concentrations of ATP.

3. Results

3.1. Expression of genes coding for adenylate cyclase isoforms

In a first step, we performed RT-PCR experiments using primers specific of each individual gene (Table 1) and RNA extracted from cultured Schwann cells. Among the nine genes encoding adenylate cyclase, only four were significantly expressed in our culture conditions, corresponding to isoforms 6, 7, 8 and 9 (Fig. 1A). Among them, two genes corresponding to isoforms 6 and 9 were the most highly expressed.

In a second step, the role of AA on the expression of these four genes was investigated. This was realized using a specific combination of primers/probes and performing quantitative PCR with extracted RNA from cell cultures grown in the absence or presence AA. In the latter case two AA concentrations were used, of 0.56 and 1.7 mM, respectively. From the data presented in Fig. 1B–E the possibility that AA acted at the transcriptional level can be excluded since the expression of none of the four genes encoding adenylate cyclase isoforms that are expressed in our cell culture conditions was affected in the presence of this molecule.

3.2. Kinetics data

The potential impact of AA on adenylate cyclase activity was then assessed in vitro using membrane preparations from Schwann cells (see Section 2) as a source of the enzyme. First the standard conditions for assaying this enzyme were investigated (Fig. 2A and B). Linearity of cAMP product formation was observed up to 10 min of reaction time, while velocity patterns versus membrane concentration were linear up to 30 μg protein in the reaction mixture. From these data, all subsequent kinetic experiments were carried out using 25 μg total protein for a reaction time of 5 min at 37 °C and varying ATP concentrations (see Section 2).

Table 1
Adenylate cyclase primer sequence

Gene	Sequence
Adcy1	5′CAGGGACGAATTCAGGTGAT3′
	5′TGCACACAAACTGGTATGAGC3′
Adcy2	5′CTGCTCGCCGTCTTCTTC3′
	5′GAACGGTTATTAAAAATGCCACA3′
Adcy3	5′GAAGATGAATCTGGAGGAGCA3′
	5′GGCAGGATGGAAAGCATAAG3′
Adcy4	5′ATGTCATTGGCCAACCA3′
	5′CTGTGATGTGCACTCGTCCT3′
Adcy5	5′TCAATGAGATCATCGCAGACTT3′
	5′CATGTAGGTGCTGCCTATGG3′
Adcy6	5′CATGATCGAAGCCATCTCG3′
	5′CCATTTCCGTAGGCCAAG3′
Adcy7	5′TGTGGGACTCTTTGGAGAATG3′
	5′CCACAAAGACGACAAACAGG3′
Adcy8	5′CTTTCAATTCCTCAGCTGTGTTTT3′
	5′CAACACCCCAGTGAAGACAA3′
Adcy9	5′TTGGGGCAATCTTGGTGT3′
	5′CAGAGCCAGTGAACATGGTG3′

Increasing concentrations of AA, from 0.2 mM up to 5 mM, resulted in an increased inhibition of adenylate cyclase activity (Fig. 3A). It is important to note that a neutralized form of AA has been used to avoid adverse effects of changing the pH value of the reaction mixture. The kinetic data were also expressed in form of double reciprocal plots (Lineweaver–Burk plots) of the reciprocal of velocity versus the reciprocal of substrate concentration (Fig. 3B). Based on these graphical representations it is apparent that the V_{max} of the enzyme was not affected by AA. In contrast the apparent K_M value was dependent on AA concentration. By linear regression analysis, all straight lines in Fig. 3B converged at the same point on the *Y*-axis, clearly indicating that AA behaves as a competitive inhibitor of adenylate cyclase under our experimental conditions. Based on secondary plots (Fig. 3C) the K_i value for the formation of the postulated enzyme–AA complex was estimated to be around 1.2 mM.

4. Discussion

The role of AA as cofactor of specific hydroxylation reactions and its antioxidant properties are well known for decades. In this context, the role of AA of the in vitro differentiation of axon-related Schwann cells was attributed to the property of this molecule to act as a cofactor in the hydroxylation of collagen proline and lysine residues, which are essential for basal lamina collagen chains to associate to triple helices [5,6]. The formation of myelin was hypothesized to be promote indirectly by AA and as a sequence of stabilized by triple-helical type IV collagen basal lamina of Schwann cells [6]. Despite intensive work, the biological properties of AA are not probably fully understood and remain to be unraveled. Recently, using an animal model of the CMT-1A, the most common form of CMT, we demonstrated that AA proved able to revert the CMT-1A phenotype [2]. This observation provided the rationale for the first phase II clinical assay of AA as a novel means to combat this disease.

The CMT-1A disorder is characterized by peripheral demyelination and has been associated with a partial duplication of the *PMP22* gene in the human chromosome 17, resulting in a

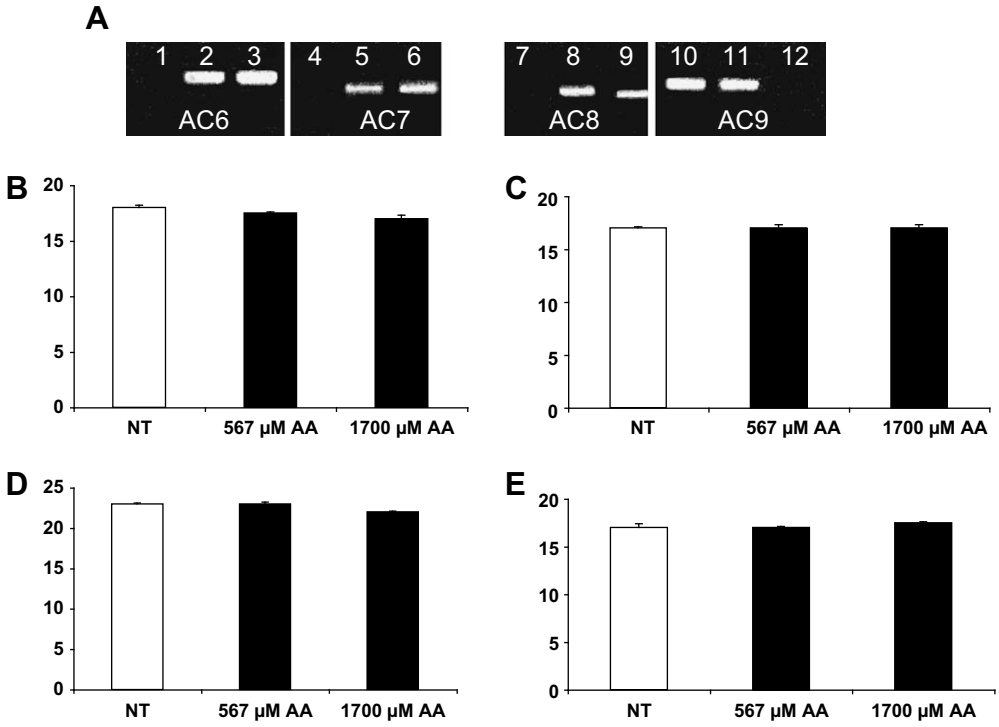

Fig. 1. (A) Adenylate cyclase gene expression. RNAs from Schwann cells have been extracted and reverse transcribed (see Section 2). Transcripts corresponding to genes coding for adenylate cyclase and expressed in Schwann cells, have been amplified (lanes 3, 6, 9 and 11). As a control of primers efficiency, we also perform RT-PCR using RNAs extracted from whole mouse embryos (lanes 2, 5, 8 and 10). We also perform RT-PCR without RNAs (lanes 1, 4, 7 and 12) as a control. (B–E) qPCR evaluation of genes respectively coding for adenylate cyclase 6, 7, 8 and 9, has been performed using specific primers. RNAs have been extracted from Schwann cells after incubation with 0.56 mM, or 1.7 mM of AA or without incubation and qPCR processed as described in methods. Level of expression is expressed with respect to the expression in cells not treated with AA (100%) (Δ Ct).

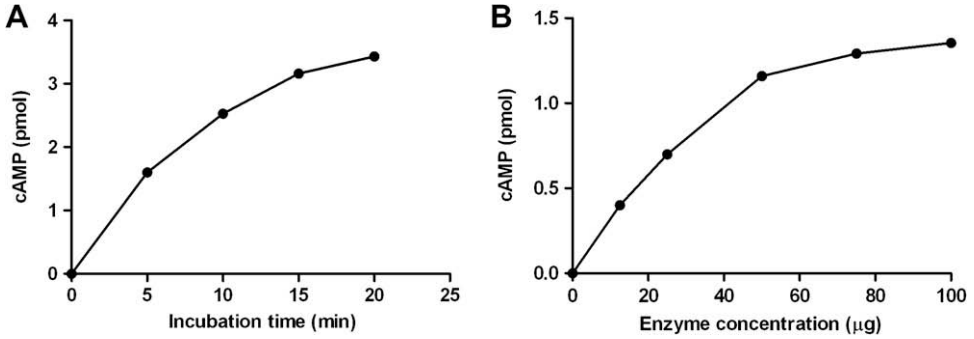

Fig. 2. Evaluation of kinetics parameters. Membranes of Schwann cells were extracted as described in Section 2 and the associated adenylate cyclase activity was evaluated using a highly specific test for the estimation of the cAMP produced by the action of the enzyme. (A) Curve of the time course of the reaction (substrate and enzyme concentrations were kept constants). (B) Curve of the reaction velocity (cAMP pmols per min) versus the enzyme concentrations (12.5 up to 100 μg total proteins) in the reaction mixture contained 1 mM ATP as substrate (total reaction time 5 min).

1.5 overproduction of the peripheral myelin protein (PMP22) possibly disturbing the native formation of myelin [7,8]. Phenotypic correction by AA might result from an action of this molecule on *PMP22* gene expression. Indeed, we previously showed that expression of this gene is under the control of the second messenger cAMP [9]. Furthermore, we recently documented that feeding Schwann cells with increasing concentrations of AA entails a lowering in the cAMP intracellular concentration [3]. In addition, we recently demonstrate that addition of tocopherol of retinol did not enhance the action of AA on the CMT1A phenotype but compete with this molecule (Kaya et al, in press). Therefore, it is obvious that AA acts on the CMT-1A phenotype by an unknown new biological function, not described so far, but different from the antioxidant property of this molecule.

In the present work, we present data that

(a) Exclude any role of AA on the expression of the genes encoding major forms of adenylate cyclase.
(b) Provide evidence that AA behaves as a competitive inhibitor of adenylate cyclase. Since this enzyme catalyses the formation of cAMP using ATP as substrate, its inhibition

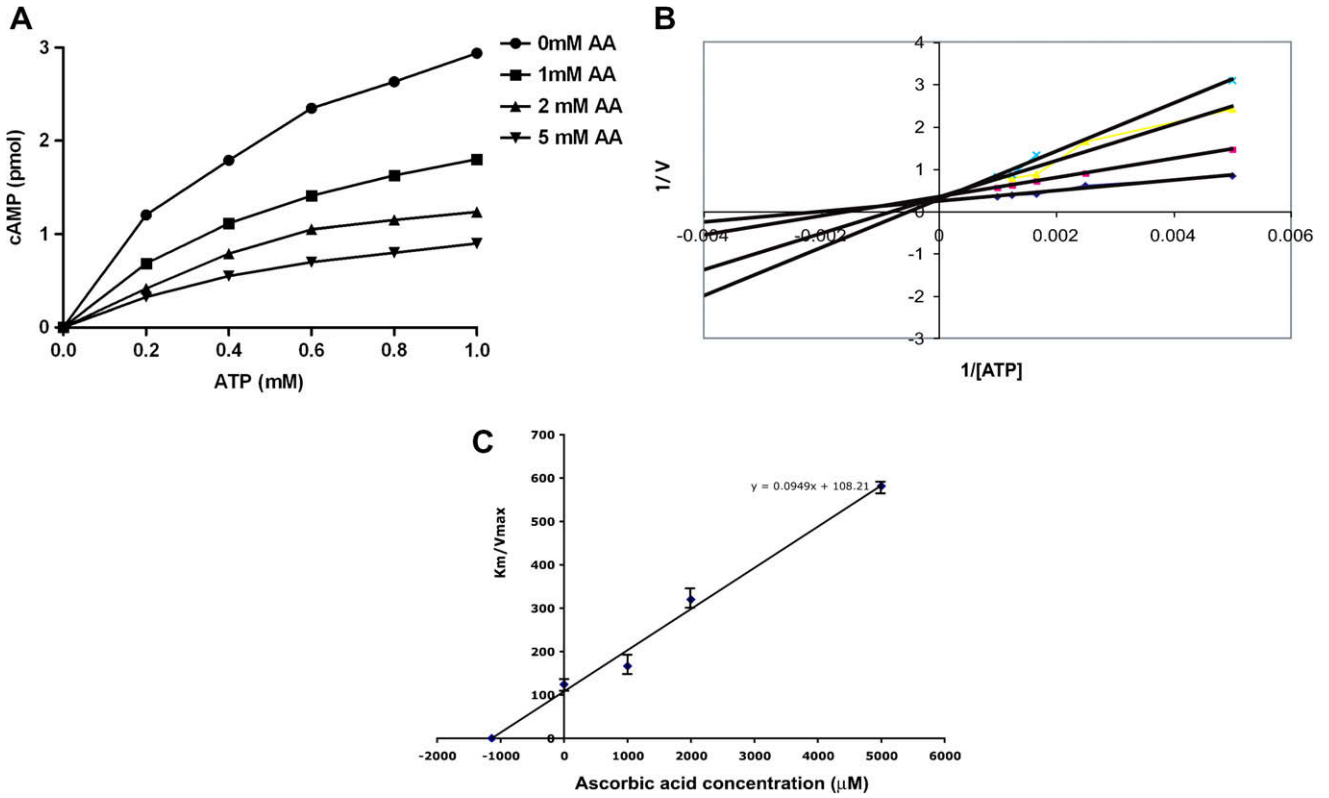

Fig. 3. Influence of ascorbic acid on adenyalte cyclase activity. Adenylate cyclase activity associated to Schwann cells membranes was studied in the absence or presence of increasing concentrations of AA up to 5 mM. (A) Michaelis–Menten representation of the variations of the reaction velocities versus ATP and ascorbic acid concentrations. (B) Lineweaver–Burk representation of the same data as in 3A. (C) A second derivative graph was drawn from the same kinetics data. A Ki of 1140 μM was estimated from that curve.

most presumably accounts for the observed decrease in cAMP concentration in cell cultures treated by AA. Hence, our present data are in general agreement with previous studies suggesting that AA acts on cAMP concentration [10,11] and on adenylate cyclase activity [12].

Considering these findings, we propose a new property of AA, which is of being a global regulator of cAMP pool by acting as a competitive inhibitor of adenylate cyclase activity. Based on our data, it is likely that AA binds to adenylate cyclase and interferes with its enzyme activity. The actual binding site is presently unknown but it is noted that the ribosyl moiety of ATP and the AA molecule share common structural properties, for example exhibiting a pentagon heterocyclic ring with an oxygen atom and two –OH groups each on the two carbons facing the oxygen atom of the ring (five-membered furan-based ring structure).

AA as a competitive inhibitor of adenylate cyclase is interesting in modulation of adenylate cyclase activity and not completely inhibiting it. This is probably fortunate for pharmacology, because a strong inhibitor will probably be toxic.

Finally, this in vitro data ask the question of the occurrence of this phenomenon in physiological conditions. In our experiments, a concentration up to 1 mM of ATP and 5 mM of AA has been used.

The intracellular concentration of ATP has been reported as variable, but is generally between 1 mM and 5 mM [13,14]. Regarding AA, a transporter, SVCT2, is involved in the intracellular transport of this molecule [15]. Therefore, the intracellular concentration of AA could be 100/200 times higher inside than outside cells, leading to intracellular concentrations ranging between 1 mM and 15 mM [16,17]. These data clearly indicate that the respective intracellular concentrations of ATP and AA that have been reported by authors are within the range (mM) of concentrations of these two molecules used in our in vitro experiments. The competitive inhibition of adenylate cyclase activity we observed in vitro, could thus happen in vivo, in physiological conditions.

In conclusion, AA could possibly be used as a pharmacological substance, acting as a competitor of adenylate cyclase, regulating by this the intracellular cAMP concentration and consequently the expression of specific genes. The discovery of this new property of AA opens new fascinating research areas concerning the exact biological function(s) of this molecule and its potential novel therapeutical properties.

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References

[1] Huxley, C., Passage, E., Manson, A., Putzu, G., Figarella-Branger, D., Pellissier, J.F. and Fontes, M. (1996) Construction of a mouse model of Charcot-Marie-Tooth disease type 1A by pronuclear injection of human YAC DNA. Hum. Mol. Genet. 5, 563–569.

[2] Passage, E., Norreel, J.C., Noack-Fraissignes, P., Sanguedolce, V., Pizant, J., Thirion, X., et al. (2004) Ascorbic acid treatment corrects the phenotype of a mouse model of Charcot-Marie-Tooth disease. Nat. Med. 10, 396–401.
[3] Kaya, F., Belin, S., Bourgeois, P., Micaleff, J., Blin, O. and Fontés, M. (2007) Ascorbic acid inhibits *PMP22* expression by reducing cAMP levels. Neuromusc. Dis..
[4] Boutry, J.M., Hauw, J.J., Gansmuller, A., Di-Bert, N., Pouchelet, M. and Baron-Van Evercooren, A. (1992) Establishment and characterization of a mouse Schwann cell line which produces myelin in vivo. J. Neurosci. Res. 32, 15–26.
[5] Carey, D.J. and Todd, M.S. (1987) Schwann cell myelination in a chemically defined medium: demonstration of a requirement for additives that promote Schwann cell extracellular matrix formation. Dev. Brain Res. 32, 95–102.
[6] Eldridge, C.F., Bunge, M.B., Bunge, R.P. and Wood, P.M. (1987) Differentiation of axon-related Schwann cells in vitro. I. Ascorbic acid regulates basal lamina assembly and myelin formation. J. Cell Biol. 105, 1023–1034.
[7] Lupski, J.R., de Oca-Luna, R.M., Slaugenhaupt, S., Pentao, L., Guzzetta, V., Trask, B.J., et al. (1991) DNA duplication associated with Charcot-Marie-Tooth disease type 1A. Cell 66, 219–232.
[8] Raeymaekers, P., Timmerman, V., Nelis, E., De Jonghe, P., Hoogendijk, J.E., Baas, F., et al. (1991) Duplication in chromosome 17p11.2 in Charcot-Marie-Tooth neuropathy type 1A (CMT-1A). The HMSN Collaborative Research Group. Neuromusc. Dis. 1, 93–97.
[9] Saberan-Djoneidi, D., Sanguedolce, V., Assouline, Z., Levy, N., Passage, E. and Fontes, M. (2000) Molecular dissection of the Schwann cell specific promoter of the *PMP22* gene. Gene 248, 223–231.
[10] Yan, J., Studer, L. and McKay, R.D. (2001) Ascorbic acid increases the yield of dopaminergic neurons derived from basic fibroblast growth factor expanded mesencephalic precursors. J. Neurochem. 76, 307–311.
[11] Doulas, N.L., Constantopoulos, A. and Litsios, B. (1987) Effect of ascorbic acid on guinea pig adrenal adenylate cyclase activity and plasma cortisol. J. Nutr. 117 (6), 1108–1114, June.
[12] Stohr, J., Novotny, J., Bourova, L. and Svoboda, P. (2005) Modulation of adenylate cyclase activity in young and adult rat brain cortex. Identification of suramin as a direct inhibitor of adenylate cyclase. J. Cell Mol. Med. 9, 940–952.
[13] Ammann, H., Noël, J., Tejedor, A., Boulanger, Y., Gougoux, A. and Vinay, P. (1995) Could cytoplasmic concentration gradients for sodium and ATP exist in intact renal cells? Can. J. Physiol. Pharmacol. 73 (4), 421–435.
[14] Fitz, J.M.D. (2007) Regulation of cellular ATP release. Trans. Am. Clin. Climatol. Assoc. 118, 166–208.
[15] Takanaga, H., Mackenzie, B. and Hediger, M.A. (2004) Sodium-dependant ascorbic acid transporter transporter family SLC23. Euro. J. Physiol. 447, 677–682.
[16] Savini, I., Rossi, A., Pierro, C., Avigliano, L. and Catani, M.V. (2008) SVCT1 and SVCT2: key proteins for vitamin C uptake. Amino Acids 34 (3), 347–355.
[17] Heideger, M.A. (2002) New view at C. Nat. Med. 8 (5), 445–446.