Potent inhibition of specific diadenosine polyphosphate hydrolases by suramin

P. Rotllán*, C.R. Rodríguez-Ferrer, A.C. Asensio, S. Oaknin

Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna, E-38206 La Laguna, Tenerife, Canary Islands, Spain

Received 17 April 1998

Abstract The cytosolic enzymes asymmetrical diadenosine tetraphosphate hydrolase (EC 3.6.1.17, Ap_4Aase) and diadenosine triphosphate hydrolase (EC 3.6.1.29, Ap_3Aase) are inhibited competitively by suramin. Ap_4Aase and Ap_3Aase were assayed in cytosolic rat brain extracts using fluorogenic analogues of the respective substrates diadenosine tetraphosphate (Ap_4A) and diadenosine triphosphate (Ap_3A). K_i values for suramin as inhibitor of Ap_4Aase and Ap_3Aase were 5×10^{-6} M and 3×10^{-7} M, respectively. Results indicate that suramin or suramin-like derivatives may be useful tools to investigate diadenosine polyphosphate cleaving enzymes and that the intracellular diadenosine polyphosphate metabolism may be a pharmacological target of suramin with biological and clinical implications.

© 1998 Federation of European Biochemical Societies.

Key words: Suramin; Diadenosine tetraphosphate; Diadenosine triphosphate; Diadenosine tetraphosphate (asymmetrical) hydrolase; Diadenosine triphosphate hydrolase; Rat brain

1. Introduction

Diadenosine polyphosphates (Ap_nA, $n=3$–6) are a family of dinucleotides with increasing biochemical and pharmacological significance as intra- and extracellular signalling molecules [1,2]. Some recent reports suggest that some Ap_nA are involved in the intracellular regulation of ATP-gated K^+ channels [3,4], in the signalling mechanisms associated with interferon actions [5] and in the mechanisms of differentiation and apoptosis in human cells [6]. These all contribute to an increasing interest in Ap_nA. Specific hydrolytic enzymes regulate the intracellular levels of these dinucleotides in mammalian cells: asymmetrical Ap_4Aase, a 20-kDa peptide, preferentially cleaves Ap_4A over Ap_5A and Ap_6A but not Ap_3A or Ap_2A, and Ap_3Aase, a 30-kDa peptide, hydrolyzes Ap_3A but not Ap_4A or Ap_2A [7,8]. It has recently been demonstrated that the protein encoded by the putative human tumor suppressor FHIT gene [9,10] displays enzymatic properties similar to those of the previously characterized Ap_3Aase.

Suramin is a symmetrical hexasulfonated naphthylamine derivative of urea used therapeutically as antiparasitic, antiviral and anticancer drug but is severely limited by toxic side effects [11,12]. Suramin is also used in the pharmacology of purinergic transmission since its effects as antagonist of P2 purinoceptors and inhibitor of ecto-ATPases [13] and neural ecto-Ap_nAase [14] have been reported. We now report inhibition of the specific Ap_4A and Ap_3A hydrolases present in rat brain cytosol by suramin, using fluorogenic derivatives of Ap_nA as substrates [15,16], and show that Ap_3Aase is a particularly sensitive enzyme.

2. Materials and methods

Young Wistar male rats were killed by decapitation, brains rapidly removed and homogenized in ice-cold 50 mM Tris-HCl pH 7.4 and centrifuged at $100\,000\times g$ for 1 h. Supernatants were dialyzed against homogenization buffer and used as Ap_4Aase and Ap_3Aase source. The fluorogenic substrates ε-(Ap_nA), $n=2$–5, were used to measure enzymatic activities by continuous fluorimetric and chromatographic assays [8,14]. Continuous assays were performed in fluorimeter microcuvettes, containing Tris-HCl 50 mM, pH 7.5, 4 mM $MgCl_2$ and appropriate protein, substrate and inhibitor concentrations (250 μl final volume) under stirring at 37°C. A Hitachi F-2000 spectrofluorimeter (λ_{ex} 305 nm, λ_{em} 410 nm) was used to record the fluorescence increase associated with substrate hydrolysis. When necessary, results of reaction rates were corrected for the quenching effect of suramin on the fluorescence emission of etheno compounds; quenching was noted at suramin concentrations higher than 5 μM. HPLC analysis was performed in a Waters instrument with a 470 fluorescence detector (λ_{ex} 305 nm, λ_{em} 410 nm). A Nova-Pak C18 column (Waters) was eluted with 10 mM KPO_4H_2, 25% acetonitrile, 2 mM tetrabutylammonium bromide (Sigma), pH 7.0 at 0.8 ml/min. Preparation of ε-(Ap_nA) was as previously described [8]. Acetonitrile and salts used for chromatographic analysis were HPLC grade from Merck and Scharlau. *C. durissus* phosphodiesterase was from Boehringer. Suramin was from RBI. All other products were reagent grade.

3. Results

3.1. Hydrolysis of ε-(Ap_nA) by rat brain extracts

The fluorogenic dinucleotides ε-(Ap_4A), ε-(Ap_5A) and ε-(Ap_3A) but not ε-(Ap_2A) were hydrolyzed by rat brain cytosolic extracts. Using both continuous fluorimetric and chromatographic assays it was found that hydrolytic activity on ε-(Ap_4A) and ε-(Ap_5A) was inhibited by Ap_4, F^-, Zn^{2+} and Ca^{2+}; however, ε-(Ap_3A) hydrolysis was not affected by Ap_4, F^-, Zn^{2+} and Ca^{2+} but by Zn^{2+} (Fig. 1A,B). The lack of ε-(Ap_2A) cleavage indicates the absence of non-specific phosphodiesterase activity able to hydrolyze Ap_nA, $n=2$–6 from brain cytosol according to known results [17]. These results

*Corresponding author. Fax: (34) (22) 630095 or (34) (22) 318354.
E-mail: protllan@ull.es

Abbreviations: Ap_nA, diadenosine 5′,5‴-P^1,P^n-polyphosphates ($n=2$–6); Ap_3A, diadenosine 5′,5‴-P^1,P^3-triphosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Ap_5A, diadenosine 5′,5‴-P^1,P^5-pentaphosphate; Ap_4, adenosine 5′-tetraphosphate; ε, etheno; ε-(Ap_nA), di-(1,N^6-ethenoadenosine) 5′,5‴-P^1,P^n-polyphosphate; ε-(Ap_2A), di-(1,N^6-ethenoadenosine) 5′,5‴P^1,P^2-pyrophosphate; ε-(Ap_3A), di-(1,N^6-ethenoadenosine) 5′,5‴-P^1,P^2-triphosphate; ε-(Ap_4A), di-(1,N^6-ethenoadenosine) 5′,5‴-P^1,P^4-tetraphosphate; ε-(Ap_5A), di-(1,N^6-ethenoadenosine) 5′,5‴-P^1,P^5-pentaphosphate; ε-Adenosine, ε-AMP, ε-ADP and ε-ATP refer to the 1,N^6-etheno derivatives of adenosine, AMP, ADP and ATP, respectively; Ap_nAases, diadenosine polyphosphate hydrolases; Ap_3Aase, diadenosine triphosphate hydrolase (EC 3.6.1.29); Ap_4Aase, asymmetrical diadenosine tetraphosphate hydrolase (EC 3.6.1.17)

0014-5793/98/$19.00 © 1998 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(98)00579-1

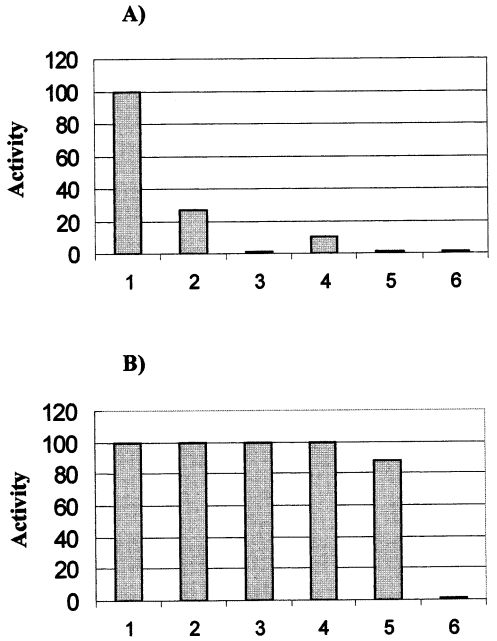

Fig. 1. Effect of several typical inhibitors of Ap_4Aase and Ap_3Aase on the hydrolysis of (A) ε-(Ap_4A) and (B) ε-(Ap_3A) by rat brain cytosolic extracts. Linear fluorescence increases were recorded from reaction mixtures for at least 5 min to determine control slopes (1) and slopes in the presence of: (2) Ca^{2+} 1 mM; (3) Ca^{2+} 6 mM; (4) F^- 200 μM; (5) Ap_4 10 μM; (6) Zn^{2+} 150 μM. Activities are expressed as percentages (means, $n=3$) of slope in the presence of inhibitor relative to control slope. Hydrolysis of ε-(Ap_5A) showed a closely similar inhibitory profile to that of ε-(Ap_4A) and hydrolysis of ε-(Ap_2A) was not detectable (not shown).

indicate that only the specific hydrolases asymmetrical Ap_4Aase and Ap_3Aase contributed to the cleavage of ε-(Ap_4A) and ε-(Ap_3A), respectively. The ε-nucleotide moieties released from ε-(Ap_4A) and ε-(Ap_3A) by asymmetrical Ap_4Aase and Ap_3Aase, ε-ATP+ε-AMP and ε-ADP+ε-AMP, respectively, were catabolized by nucleotidases and/or phosphatases up to ε-adenosine as the final product (Fig. 2A,B). K_m and specific activity values obtained for Ap_4Aase and Ap_3Aase were respectively: 2.1 ± 0.4 and 11.2 ± 1.7 μM, and 10.5 ± 1.8 and 2.3 ± 0.6 nmol/min/mg ($n=4$).

3.2. *Inhibition of asymmetrical Ap_4Aase and Ap_3Aase by suramin*

Addition of suramin (0.1–30 μM) to the reaction mixtures in the fluorimeter cuvette produced immediate and sustained decreases of the slope of fluorescence traces associated with substrate hydrolysis. The slope changes induced by suramin were dose dependent and were not modified after preincubation of suramin in the reaction mixture. HPLC assays clearly demonstrated that the depressor effect of suramin on fluorescence trace slopes was associated with a decrease in the substrate cleavage efficiency (Fig. 2C,D).

Fig. 3 shows comparatively the effects of suramin on Ap_4Aase and Ap_3Aase. Ap_3Aase was found to be the most sensitive enzyme showing a complete inhibition at suramin concentrations higher than 6 μM. Increased substrate concentration in the reaction mixture attenuated suramin inhibition, suggesting competition between substrate and inhibitor at the binding site. Double reciprocal plots obtained for Ap_4Aase and Ap_3Aase were compatible with a competitive inhibition pattern by suramin (Fig. 4). K_i values determined from these plots were 5.03 ± 0.91 μM and 0.31 ± 0.04 μM for Ap_4Aase and Ap_3Aase, respectively, with K_m/K_i quotients of 0.4 and 36. A K_m/K_i quotient of 36 indicates that Ap_3Aase exhibits much greater affinity for suramin than for its own substrate Ap_3A, although this is not the case for Ap_4Aase.

4. Discussion

Rat brain cytosolic activities hydrolyzing the fluorogenic substrates of Ap_4A and Ap_3A display the typical characteristics of asymmetrical Ap_4Aase and Ap_3Aase from bovine adrenomedullary tissue and various other mammalian sources [7,8]. Both K_m and specific activities determined for these specific hydrolases compare well with those determined for enzymes from various rat organs, including the brain using Ap_4A and Ap_3A as substrates [8].

These K_i values for suramin as inhibitor of specific Ap_nAases are within the range of lower K_i values reported

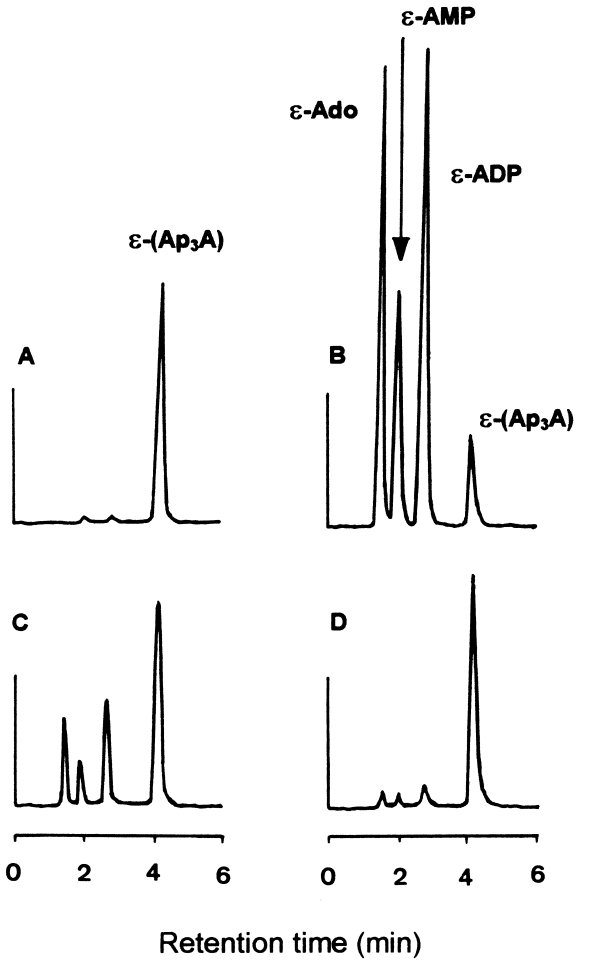

Fig. 2. Chromatographic profiles demonstrating the hydrolysis of ε-(Ap_3A) by rat brain cytosolic extracts and the inhibitory effect of suramin on the dinucleotide hydrolysis. 5-μl aliquots from reaction mixtures of 100 μl containing 10 μM ε-(Ap_3A) and incubated at 37°C were taken at 0 and 20 min and injected into the chromatograph. A: Control, 0 min. B: Hydrolysis control without suramin, 20 min. C: Hydrolysis in the presence of 2 μM suramin, 20 min. D: Hydrolysis in the presence of 6 μM suramin, 20 min. Inhibition of ε-(Ap_4A) hydrolysis by suramin was also demonstrated by similar experiments (not shown).

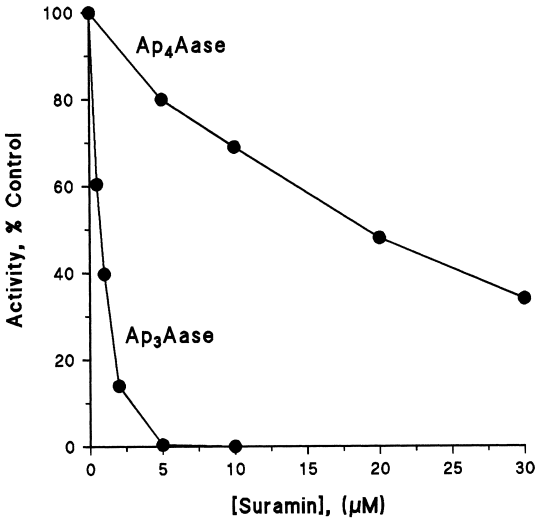

Fig. 3. Suramin inhibition of specific Ap_nAases. Cytosolic extracts were incubated as described in Section 2, in a final volume of 250 μl in the presence of 10 μM ε-(Ap_4A) or ε-(Ap_3A) and at increasing suramin concentrations. Linear fluorescence increases were recorded for 5 min. Activities are expressed as percentages (means, $n=3$) of activity measured in control reaction mixtures in the absence of suramin.

for most suramin-sensitive enzymes [11,14,18,19]. Competitive inhibition for Ap_4Aase and Ap_3Aase suggests that suramin, a polyanionic molecule, interacts primarily by electrostatic interactions with positively charged amino acid residues involved in the binding of the negatively charged substrates Ap_4A and Ap_3A. Hydrophobic interactions due to suramin polyaromatic rings and hydrogen bonding probably also contribute to suramin binding. Other enzymes using nucleotide substrates, e.g. ATP, like human folylpolyglutamate synthetase [18] or a *Xenopus* oocyte ecto-ATPase [20] have been reported to be competitively inhibited by suramin. We have tentatively proposed that lysine is not involved in substrate

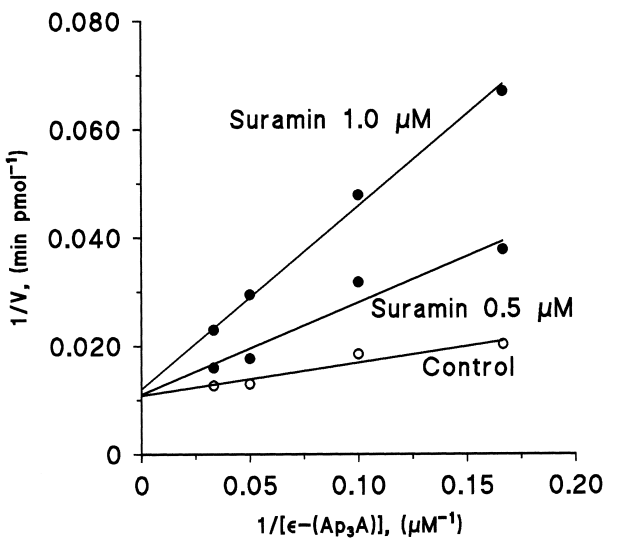

Fig. 4. Double reciprocal plot of Ap_3Aase inhibition by suramin. Cytosolic extracts were prepared and incubated as described in Section 2 in a final volume of 250 μl at several substrate concentrations in the absence (control) and presence of suramin 0.5 and 1.0 μM. The graph represents the mean of three experiments. A similar competitive pattern (not shown) was obtained for Ap_4Aase.

binding in bovine adrenomedullary Ap_4Aase [8]; consequently histidine and/or arginine could be involved in the binding of Ap_4A to Ap_4Aase. The complete inactivation of both asymmetrical Ap_4Aase and Ap_3Aase observed after preincubation of brain extracts with diethyl pyrocarbonate (unpublished observations) strongly suggests the presence of catalytically active histidine residues in both enzymes.

The similarity between the catalytic properties of FHIT protein [9] and Ap_3Aase (EC 3.6.1.29), an enzyme known for years [7], suggests that both could be the same protein. If this is so, it may then be predicted that suramin will be a FHIT protein-binding drug inhibiting its associated Ap_3Aase activity, which opens the question of the biological activity displayed by putative FHIT-suramin complexes.

Inhibition of Ap_4Aase and Ap_3Aase by suramin may have clinical and biological relevance. When used therapeutically, suramin serum concentrations in treated patients may be higher than 200 μM with a measured half-life of about 50 days and it has been reported that the drug may inhibit in vivo cytosolic, lysosomal, and nuclear enzymes [11,12,21,22]. Thus, suramin inhibition of Ap_nA hydrolases is a feasible event that could contribute partly to the great diversity of toxic effects described for suramin by increasing cellular Ap_nA levels.

Raised intracellular Ap_nA levels caused by suramin inhibition of both Ap_nA hydrolases could interfere with cellular energy metabolism through the very strong inhibition of adenosine and adenylate kinases by Ap_4A and Ap_5A [23,24] or with the normal regulation of ATP-gated K^+ channels, which are also Ap_nA-binding proteins [3,4]. It is worth noting that increased Ap_3A levels have recently been correlated with cellular differentiation in responsive human cells treated by interferons [5,6] and that suramin can induce cell differentiation [25]. This raises the question whether suramin induced differentiation could be mediated by increased Ap_3A levels or changes in the Ap_3A/Ap_4A ratio, a recently proposed sensitive indicator of cell status [6].

This study adds asymmetrical Ap_4Aase and Ap_3Aase to the list of enzymes inhibited by suramin. Since there are no suitable inhibitors of these enzymes, our results suggest that suramin or suramin-derived compounds more specifically inhibiting these Ap_nA hydrolases could be tools to further investigate molecular aspects of Ap_nA-cleaving enzymes including FHIT protein, and also the intracellular roles of Ap_nA, still a puzzling question far from being clearly understood.

Acknowledgements: This work was supported by Grant PM96-0081.

References

[1] Baxi, M.D. and Vishwanatha, J.K. (1995) J. Pharmacol. Toxicol. Methods 33, 121–128.
[2] Pintor, J., Hoyle, C.H.V., Gualix, J. and Miras-Portugal, M.T. (1997) Neurosci. Res. Commun. 20, 69–78.
[3] Jovanovic, A., Alekseev, A.E. and Terzic, A. (1997) Biochem. Pharmacol. 54, 219–225.
[4] Ripoll, C., Martin, F., Rovira, J.M., Pintor, J., Miras-Portugal, M.T. and Soria, B. (1996) Diabetes 45, 1431–1434.
[5] Vartanian, A., Narovlyansky, A., Amchenkova, A., Turpaev, K. and Kisselev, L. (1996) FEBS Lett. 381, 32–34.
[6] Vartanian, A., Prudovsky, I., Suzuki, H., Pra, I.D. and Kisselev, L. (1997) FEBS Lett. 415, 160–162.
[7] Guranowski, A. and Sillero, A. (1992) in: Ap4A and Other Di-

nucleoside Polyphosphates (McLennan, A.G., Ed.) pp. 81–133, CRC Press, Boca Ratón, FL.
[8] Ramos, A. and Rotllán, P. (1995) Biochim. Biophys. Acta 1253, 103–111.
[9] Barnes, L.D., Garrison, P.N., Siprashvili, Z., Guranowski, A., Robinson, A.K., Ingram, S.W., Croce, C., Ohta, M. and Huebner, K. (1996) Biochemistry 35, 11529–11535.
[10] Siprashvili, Z., Sozzi, G., Barnes, L.D., McCue, P., Robinson, A.K., Eryomin, V., Sard, L., Tagliabue, E., Greco, A., Fusetti, L., Schwartz, G., Pierotti, M.A., Croce, C.M. and Huebner, K. (1997) Proc. Natl. Acad. Sci. USA 94, 13771–13776.
[11] Voogd, T.E., Vansterkenburg, E.L.M., Wilting, J. and Janssen, L.H. (1993) Pharmacol. Rev. 45, 177–203.
[12] Baghdiguian, S. and Fantini, J. (1997) Cancer J. 10, 31–37.
[13] Windscheif, U. (1996) J. Pharm. Pharmacol. 48, 993–1011.
[14] Mateo, J., Rotllán, P. and Miras-Portugal, M.T. (1996) Br. J. Pharmacol. 119, 1–2.
[15] Wierzchowski, J., Sierakowska, H. and Shugar, D. (1985) Biochim. Biophys. Acta 82, 109–115.
[16] Rotllán, P., Ramos, A., Pintor, J., Torres, M. and Miras-Portugal, M.T. (1991) FEBS Lett. 280, 371–374.
[17] García-Agúndez, J.A., Cameselle, J.C., Costas, M.J., Günther, M.A. and Sillero, A. (1991) Biochim. Biophys. Acta 1073, 402–409.
[18] McGuire, J.J. and Haile, W.H. (1996) Arch. Biochem. Biophys. 335, 139–144.
[19] Cadène, M., Duranton, J., North, A., Si-Tahar, M., Chignard, M. and Bieth, J.G. (1997) J. Biol. Chem. 272, 9950–9955.
[20] Ziganshin, A.U., Ziganshina, L.L., King, B.F. and Burnstock, G. (1995) Pflügers Arch. Eur. J. Physiol. 429, 412–418.
[21] La Rocca, R.V., Stein, C.A. and Myers, C.E. (1990) Cancer Cells 2, 106–115.
[22] Bojanowski, K., Lelièvre, S., Markovits, J., Couprie, J., Jacquemin-Sablin, A. and Kragh-Larsen, A. (1992) Proc. Natl. Acad. Sci. USA 89, 3025–3029.
[23] Rotllán, P. and Miras-Portugal, M.T. (1985) Eur. J. Biochem. 151, 365–371.
[24] Lienhard, G.E. and Secemsky, I.I. (1973) J. Biol. Chem. 248, 1121–1123.
[25] Fantini, J., Rognoni, J., Roccabianca, M., Pommier, G. and Marvaldi, J.J. (1989) J. Biol. Chem. 264, 10282–10286.