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A novel assay for 5′-nucleotidase using 1,N^6-etheno-AMP as substrate, and comments on the properties of the reaction product, ethenoadenosine

Zahirali JAMAL, Amir AFKHAM-EBRAHIMI and E. David SAGGERSON
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

A novel assay for 5′-nucleotidase is described in which 1,N^6-etheno-AMP is converted into ethenoadenosine. The product ethenoadenosine is neither a substrate for nor an inhibitor of adenosine deaminase. Ethenoadenosine appears to have little effect at adenosine receptors on adipose-tissue cells.

INTRODUCTION

5′-Nucleotidase (EC 3.1.3.5) is an ectoenzyme in many tissues concerned with the conversion of AMP into adenosine. This activity is probably part of a metabolic pathway for removing extracellular adenine nucleotides released during processes such as neurotransmission (Burnstock, 1981), strenuous exercise (Forrester & Lind, 1969), platelet thrombus formation (Gaarder *et al.*, 1961) and shock (Trams *et al.*, 1980). In turn, besides being further metabolized, the product adenosine has effects at A_1- or A_2-type adenosine receptors on various tissues, including vasculature [a possible role of 5′-nucleotidase in the regulation of blood flow has been proposed for several years; see, e.g., Baer *et al.* (1966); Baer & Drummond (1968); Nakatsu & Drummond (1972)]. 5′-Nucleotidase is interesting in other respects. As a cell-surface glycoprotein it undergoes various stages of post-translational processing (Wada *et al.*, 1986; van den Bosch *et al.*, 1986), appears to circulate between the cell surface and an intracellular pool (Stanley *et al.*, 1980; Wilcox *et al.*, 1982; Widnell *et al.*, 1982), possibly interacts with elements of the cytoskeleton (Mannherz & Rohr, 1978; Carraway *et al.*, 1979), and is attached to the plasma membrane either as a short-stalked integral membrane protein (Baron *et al.*, 1986) or through a glycosyl-phosphatidylinositol lipid anchor (Low *et al.*, 1986; Low, 1987). In addition, in white adipose tissue 5′-nucleotidase activity shows sex differences and adaptivity in several pathophysiological states (Green *et al.*, 1981; Vernon *et al.*, 1983; Newsholme *et al.*, 1985; Jamal & Saggerson, 1987).

Over the past two decades 5′-nucleotidase has been assayed in various ways. These include measurement of orthophosphate release (colorimetric or radiochemical; Widnell, 1974), spectrophotometric measurement by coupling to adenosine deaminase (Widnell & Unkeless, 1968; Belfield & Goldberg, 1968; Burger & Lowenstein, 1970), or measurement of the conversion of radiolabelled AMP into adenosine. In the last method, product and substrate have been separated by ion-exchange chromatography (Glastris & Pfeiffer, 1974), by paper chromatography (Widnell & Unkeless, 1968) or by precipitation of AMP with $ZnSO_4 + Ba(OH)_2$ (Avruch & Wallach, 1971; Newby *et al.*, 1975).

Here we demonstrate a novel simple assay of 5′-nucleotidase that exploits the fluorescent properties of etheno-AMP and ethenoadenosine, and we also make some comments on the metabolism and pharmacological action of ethenoadenosine.

MATERIALS AND METHODS

Chemicals

These were obtained and treated as described by Woodward & Saggerson (1986) and Jamal & Saggerson (1987). In addition, 1,N^6-ethenoadenosine, 1,N^6-etheno-5′-AMP and concanavalin A were from Sigma Chemical Co., Poole, Dorset, U.K.

Isolation of adipocytes

White adipocytes were isolated from the epididymal adipose tissues of male Sprague–Dawley rats (160–180 g) essentially as described by Rodbell (1964). Brown adipocytes were isolated from the interscapular depot of the same animals (Woodward & Saggerson, 1986) by the procedure originally described by Fain *et al.* (1967) and elaborated by Nedergaard & Lindberg (1982).

Preparation of white-adipocyte extracts

Preparations of white adipocytes from two rats were washed twice in 10 ml of buffer (45 mM-Tris/HCl/45 mM-β-glycerophosphate, pH 7.4). The cells were then resuspended in 10 ml of the same ice-cold buffer and homogenized at 500 rev./min by five up-and-down strokes in a Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle (radial clearance 0.2 mm). The homogenate was first centrifuged at 300 $g_{av.}$ for 1 min at 4 °C to remove fat. Portions of this defatted homogenate were stored frozen at −40 °C, and the remainder was re-centrifuged for 30 min at 30000 $g_{av.}$ to yield a 'particulate protein' pellet, which was re-suspended in 1 ml of the Tris/β-glycerophosphate buffer and also stored at −40 °C. Protein contents of extracts were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Assays for 5′-nucleotidase

These were based on the method of Newby *et al.* (1975) and were performed at 37 °C in a shaking water bath (approx. 100 cycles/min). The final volume of 0.5 ml contained 50 mM-Tris/HCl buffer (pH 8.0) and appropriate concentrations of [2-^{3}H]AMP (0.6 μCi/

μmol) or etheno-AMP. The reactions were started by addition of 20–50 μg of 30000 *g* particulate protein or 50–100 μg of defatted adipocyte homogenate, and were carried out over 5–15 min. Termination was by addition of 0.1 ml of ice-cold 0.15 M-$ZnSO_4$, followed immediately by 0.1 ml of 0.15 M-$Ba(OH)_2$. The tube contents were mixed vigorously, stored on ice for 10 min, and then centrifuged for 10 min at 6500 $g_{av.}$ in an Eppendorf 5412 centrifuge. This procedure separates the precipitated [^{3}H]AMP or etheno-AMP in the pellet from the nucleoside products, which are retained in the supernatant. When [^{3}H]AMP was the substrate, 0.5 ml of the supernatant was mixed with 13 ml of scintillant [consisting of 80 g of naphthalene plus 4 g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen in 600 ml of toluene plus 400 ml of 2-methoxyethanol] and taken for liquid-scintillation counting. When etheno-AMP was the substrate, fluorescence of 0.5 ml of the supernatant was read in a Perkin–Elmer 3000 fluorimeter at an excitation wavelength of 275 nm and an emission wavelength of 410 nm. The fluorimeter was calibrated against a standard curve of ethenoadenosine (1–16 μM).

Assay of adenosine deaminase

Calf intestine adenosine deaminase (EC 3.5.4.4) supplied in 3.2 M-$(NH_4)_2SO_4$ was centrifuged for 2 min at 6500 $g_{av.}$ in an Eppendorf 5412 centrifuge and dissolved in 10 vol. of 0.15 M-NaCl. The enzyme was assayed spectrophotometrically at 265 nm and 25 °C in 0.1 M-glycylglycine buffer (pH 7.4) as described by Kalckar (1947), with additions of adenosine or ethenoadenosine as appropriate.

Experiments with isolated adipocytes

Measurements of white-adipocyte lipolysis and brown-adipocyte O_2 uptake were made as described by Saggerson (1986) and Woodward & Saggerson (1986) respectively.

T.l.c.

Ethenoadenosine was separated from etheno-AMP on Kieselgel 60 F_{254} plates in a solvent system consisting of propan-2-ol/ethyl acetate/8 M-NH_3 (9:4:3, by vol.) (Arch & Newsholme, 1978).

Spectra

Absorption spectra and fluorescence spectra were obtained with a Pye–Unicam SP8-100 spectrophotometer and a Perkin–Elmer 3000 fluorimeter respectively.

RESULTS AND DISCUSSION

Assay of 5′-nucleotidase using etheno-AMP as substrate

It was found that the fluorescent analogue etheno-AMP could be conveniently substituted for [^{3}H]AMP in the assay initially devised by Newby *et al.* (1975). In preliminary experiments (results not shown), with either defatted homogenates or 30000 *g* particulate fractions from white adipocytes, it was established that production of fluorescent material not precipitated by $ZnSO_4 + Ba(OH)_2$ was linear both with protein concentration and with time for at least 15 min. T.l.c. of the supernatant from such assays showed the formation of a single detectable product ($R_F = 0.46$) which appeared to be identical with chromatographed standard amounts of ethenoadenosine. The background in the assay was extremely small, since 99.7% of 200 μM-etheno-AMP was precipitated by $ZnSO_4 + Ba(OH)_2$. On the other hand, when samples containing 1–10 μM-ethenoadenosine were treated with $ZnSO_4 + Ba(OH)_2$, negligible precipitation of fluorescent material was observed in the presence or the absence of adipocyte protein. Using 30000 *g* particulate material as enzyme source, the rate

Table 1. Comparison of 5′-nucleotidase assays with [^{3}H]AMP and etheno-AMP as substrate

Assays were performed for 15 min with adipocyte 30000 *g* particulate protein. K_m values were determined from Lineweaver–Burk plots with 5, 10, 20, 35, 50, 75, 100, 150, 200, 250 and 300 μM substrate. The values are means ± S.E.M. for the numbers of separate preparations shown in parentheses.

	Assay substrate	
	[^{3}H]AMP	Etheno-AMP
K_m (μM)	42.3 ± 2.8 (3)	25.0 ± 1.8 (4)
Activity at 200 μM substrate (nmol/min per mg of protein)	23.5 ± 2.0 (4)	6.6 ± 0.4 (4)

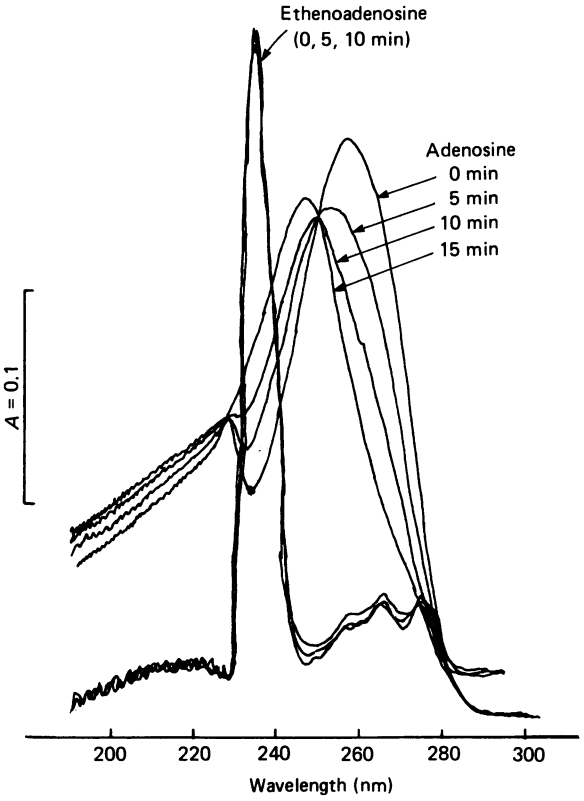

Fig. 1. Absorption changes on incubation of adenosine and ethenoadenosine with adenosine deaminase

Adenosine or ethenoadenosine (150 μM) was incubated for the indicated times in 3.0 ml of 0.1 M-glycylglycine buffer (pH 7.4) with 0.2 unit of calf intestine adenosine deaminase at 25 °C.

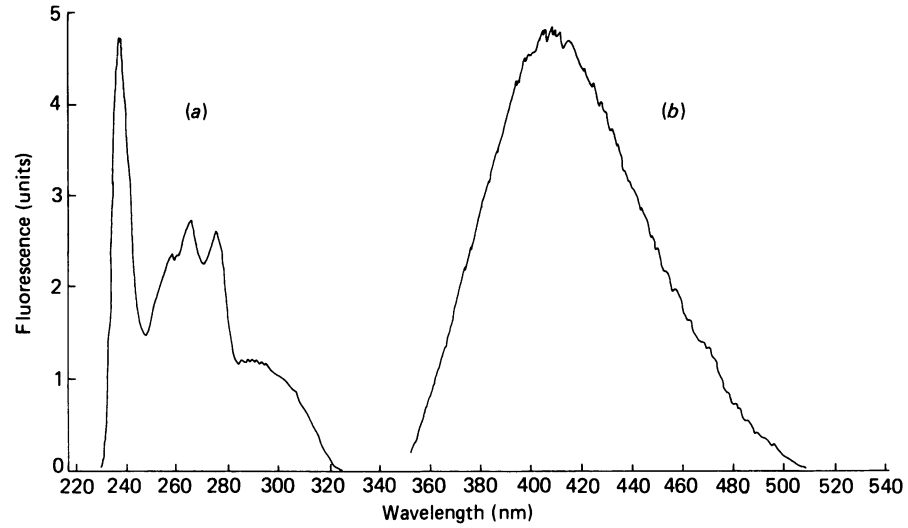

Fig. 2. Fluorescence spectra of ethenoadenosine after incubation with adenosine deaminase

Ethenoadenosine (150 μM) was incubated for 30 min at 25 °C in 3.0 ml of 0.1 M-glycylglycine buffer (pH 7.4) with 0.2 unit of calf intestine adenosine deaminase. (*a*) Excitation spectrum, emission measured at 410 nm. (*b*) Emission spectrum, excitation at 235 nm. Both spectra are identical with those observed before the addition of adenosine deaminase.

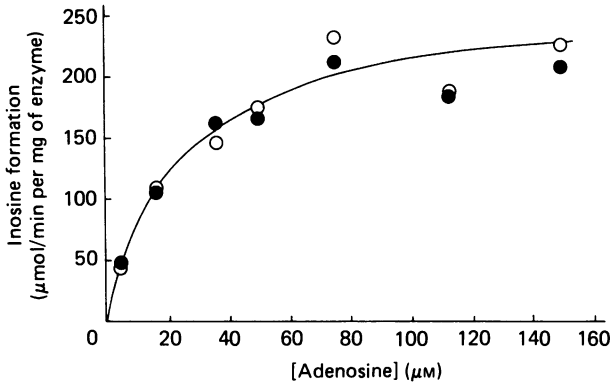

Fig. 3. Activity of adenosine deaminase in the presence and absence of ethenoadenosine

Adenosine deaminase activity was assayed at 25 °C as described in the Materials and methods section by using 0.2 μg of calf intestine enzyme and the indicated concentrations of adenosine: ○, ●, without and with 40 μM-ethenoadenosine respectively.

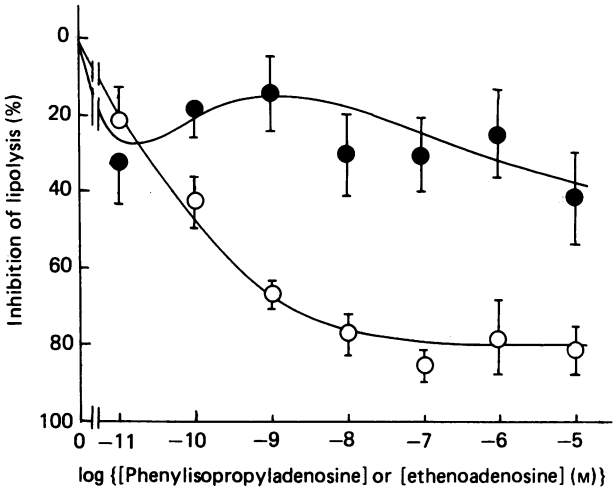

Fig. 4. Comparison of effects of enthoadenosine and phenylisopropyladenosine on white-adipocyte lipolysis

Adipocytes were incubated for 1 h in Krebs–Ringer bicarbonate medium containing 5 mM-glucose, albumin (40 mg/ml), adenosine deaminase (1 unit/ml), 0.1 μM-noradrenaline and the indicated concentrations of nucleoside derivations: ○, with phenylisopropyladenosine ($n = 4$); ●, with ethenoadenosine ($n = 6$). The values are means ± S.E.M. for n separate cell preparations.

of conversion of 200 μM-etheno-AMP into ethenoadenosine was inhibited by 10 μM-concanavalin A by 89%, which is characteristic of membrane-associated 5′-nucleotidase (Riordan & Slavik, 1974; Reimer & Widnell, 1975). In our hands, with rat adipocyte material as enzyme source, the fluorescence assay was sensitive enough to detect the formation of 500 pmol of ethenoadenosine. Table 1 shows that the apparent K_m of the adipocyte particulate 5′-nucleotidase for etheno-AMP was appreciably lower than for the physiological substrate AMP. However, the intrinsic activity, with a near-saturating concentration (200 μM) of the fluorescent substrate, was only 26% of that with AMP.

Although commerically etheno-AMP is more expensive than the equivalent amount of AMP (+[^{3}H]AMP), the fluorescence assay has the advantage of speed and simplicity. A very large number of samples can be read with a fluorimeter in a short time. No radioactive sample preparation or liquid-scintillation-counting time is needed. A second possible advantage of the fluorescence assay is that further metabolism of the product, ethenoadenosine, is restricted and its pharmacological effects may be less than those of adenosine.

Ethenoadenosine and adenosine deaminase

Adenosine deaminase is present at quite high activity in most tissues, including blood, and represents a potental

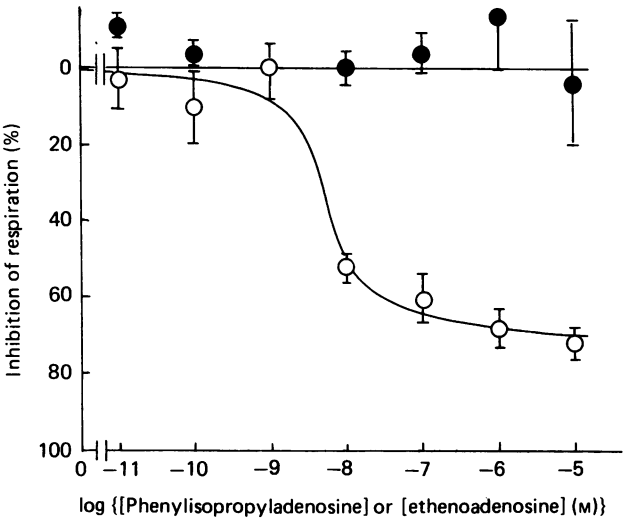

Fig. 5. Comparison of effects of ethenoadenosine and phenylisopropyladenosine on brown-adipocyte respiration

O_2 uptake was measured in cells incubated in Krebs–Ringer bicarbonate medium containing 5 mM-glucose, albumin (40 mg/ml), adenosine deaminase (1 unit/ml), 10 nM-noradrenaline and the indicated concentrations of nucleoside derivatives. The values are means ± S.E.M. for four separate cell preparations; symbols as for Fig. 4.

problem when 5′-nucleotidase is assayed in crude systems, since adenosine, inosine, hypoxanthine, xanthine and even uric acid may appear as products of the assay. With etheno-AMP as substrate it is perfectly feasible to assay 5′-nucleotidase activity in whole homogenates (see above), and it should be possible to assay the ectoenzyme reliably in cell suspensions or even in tissue perfusions. This conclusion is based on the following findings.

After incubation of 200 μM-ethenoadenosine with calf intestine adenosine deaminase, the only spot detected on t.l.c. was ethenoadenosine (results not shown). In addition, whereas deamination of adenosine by adenosine deaminase produced characteristic changes in absorption spectrum (Fig. 1), there was no change in the absorption spectrum of ethenoadenosine between 190 and 300 nm when adenosine deaminase was added (Fig. 1). Similarly, neither the fluorescence excitation nor the emission spectrum changed on treatment of ethenoadenosine with adenosine deaminase (Fig. 2). Fig. 3 shows that ethenoadenosine had no appreciable effect on adenosine deaminase activity at any tested concentration of the physiological substrate for this enzyme. It was therefore concluded that ethenoadenosine is neither a substrate nor an inhibitor of adenosine deaminase.

Effects of ethenoadenosine at adipocyte adenosine receptors

If etheno-AMP were to be used to assay 5′-nucleotidase in whole-tissue preparations, it is of use to know whether the resulting ethenoadenosine has effects at tissue adenosine receptors which could cause extensive changes in tissue metabolism. Adenosine is antilipolytic in white and brown adipose tissues, and decreases O_2 uptake in brown adipocytes (Saggerson, 1986; Woodward & Saggerson, 1986), and these effects appear to be mediated through A_1-type receptors. Fig. 4 shows that the A_1-receptor agonist phenylisopropyladenosine inhibited up to 85% of the lipolysis elicited by 0.1 μM-noradrenaline, with an IC_{50} (concn. giving 50% inhibition) of approx. 0.1 nM. By contrast, ethenoadenosine had only small effects, and the decrease in lipolysis was never more than 40%. Fig. 5 shows that phenylisopropyladenosine decreased by 70% the brown-adipocyte respiration elicited by 10 nM-noradrenaline, with an IC_{50} of approx. 5 nM. By contrast, ethenoadenosine had no effect at any tested concentration. In addition (results not shown), 1 μM-ethenoadenosine did not affect the IC_{50} for phenylisopropyladenosine, suggesting that ethenoadenosine does not act as an antagonist at the A_1 receptors in brown adipocytes.

Conclusions

We propose a novel and simple assay for 5′-nucleotidase which has the advantages that the product, ethenoadenosine, is not further metabolized by adenosine deaminase and, in adipose tissue at least, has little effect at adenosine receptors.

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