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Inhibition of soluble 5′-nucleotidase from rat brain by different xanthine derivatives

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There is increasing evidence that adenosine plays a role in the regulation of brain function [1–3]. Similarly there is good evidence that xanthine derivatives, such as theophylline and caffeine, may exert many of their actions due to adenosine receptor antagonism [2–4]. However, xanthine derivatives may affect not only the actions of adenosine, but also its formation. Thus it has been shown that xanthine derivatives are able to inhibit 5′-nucleotidase [5–7].

5′-Nucleotidase is present both as a soluble and as a membrane bound ecto-enzyme [8]. The latter form of the enzyme catalyzes the breakdown of AMP present on the outside of cells, and may be of importance in the salvage of purines from 'lost' purine nucleotides [9]. The intracellular, soluble form of the enzyme may be involved in the formation of intracellular adeonsine from AMP. Intracellular formation of adenosine may be of quantitatively greater importance than the formation from extracellular adenine nucleotides [10–12].

Whereas the earlier studies [5–7] demonstrated that the membrane bound form of the enzyme is inhibited by xanthine derivatives, the present study reports that the soluble enzyme also is inhibited by several xanthine derivatives. The structural requirements differ from those for adenosine receptor antagonism or phosphodiesterase inhibition.

Materials and methods

Male Sprague–Dawley rats (200 g) were killed by guillotine. The brains were rapidly dissected out, placed in ice-cooled saline and homogenized in 6 vol. of 0.25 M sucrose containing 50 mM Tris–Cl pH 7.4, 1 mM EDTA and 10 mM 2-mercaptoethanol. After an initial centrifugation at 800 *g* for 20 min to remove cell debris, the supernatant was centrifuged at 100,000 *g* for 45 min. The supernatant served as the source of soluble enzyme, the resuspended pellet (6 ml) as the source of membrane bound enzyme. The enzyme was stored frozen at −80°C in aliquots.

5′-Nucleotidase activity was assayed using ^{14}C-AMP as substrate by the method described earlier [5]. 50 μl suitably diluted enzyme was incubated for 5–60 min in a total volume of 1 ml of 50 mM Tris–Cl pH 7.8 containing 1 mM Mg acetate and labelled AMP as well as other additions as indicated. The assay was done at 30°C and was stopped by the addition of Zn-sulfate and Ba-hydroxide. Blanks were fortified with 30–50 μM α,β methylene ADP [13].

The particulate enzyme was diluted 25-fold and the assay was conducted for 30 min. The supernatant enzyme was used undiluted and the incubations lasted for 60 min. Under these conditions the assay was linear with time.

The conversions of ^{14}C-AMP and of ATP during incubation with the supernatant fraction was determined by HPLC as described earlier [20, 21].

The following drugs were used: 8-^{14}C-adenosine 5′-monophosphate (549 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. Theophylline and caffeine were obtained from ACO, Göteborg, Sweden; Xanthine, 1,7-dimethylxanthine, 7-methylxanthine, 3-methylxanthine, adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP) were from Sigma, St. Louis, MO; Verrophylline (3,7-dihydro-1,8-dimethyl-3-(2-methylbutyl)-1H-purine-2,6-dione) was a gift from Dr K. Murphy, Johns Hopkin, Baltimore, MD; Theobromine was from Astra, Södertälje, Sweden; 3-isobutyl-1-methylxanthine (IBMX) was from Aldrich Co., Rahaway, NJ; 7-benzyl-3-isobutyl-1-methylxanthine was synthesized by Dr G. Kjellin, AB Draco, Lund, Sweden, who also supplied enprofylline (3,7-dihydro-3-1H-purine-2,6-dione); 8-sulphophenyl-theophylline was a gift from Dr G.F. Bruns, Warner Lambert Co., Ann Arbor, MI.; 8-phenyl-theophylline from Calbiochem-Behring Co., CA. and 1,3-diethyl-8-phenylxanthine from Research Biochemicals Inc., Wayland MA were dissolved in a 1 mM solution of tetraphenylbromate; Pentoxiphylline and 1-(5′-oxohexyl)-3-methyl-7-propylxanthine (HWA 285) were gifts from Dr R. Sehleyerbach of Hoechst Ag., Wiesbaden, FRG; α,β-methylene adenosine 5′-diphosphate (AOPCP) was from P.L. Biochemicals, Milwaukee, WI.

Results and discussion

Approximately one fourth of the total 5′-nucleotidase

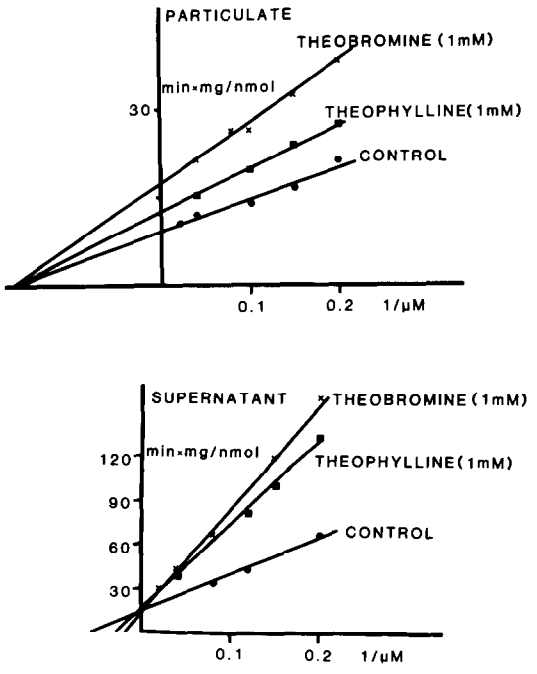

Fig. 1. The effect of theophylline and theobromine on the hydrolysis of AMP in a soluble and particulate fraction of the rat brain. The results are given as a Lineweaver–Burk plot.

activity was present in the supernatant after centrifugation. This fraction is lower than that reported by Montero and Fez [15] for bovine brain, but much higher than reported by Song and Bodanski [16] for rat liver. The kinetics of the 5′-nucleotidase, is shown in Fig. 1. In this experiment an apparent K_m of 17 μM was found. The results from five separate experiments gave an average K_m of 12.2 ± 2.1 μM and the average V_{max} of 107 ± 21 pmol/min per mg protein (mean ± S.E.M.).

In these experiments we used the competitive inhibitor of 5′-nucleotidase, AOPCP, to define the 5′-nucleotidase activity, as suggested by Burger and Lowenstein [17] and by Gentry and Olsson [13]. ATP, which is commonly assumed to be the most important endogenous inhibitor of the enzyme [8], caused a higher degree of inhibition of ^{14}C-AMP hydrolysis than AOPCP. However, the inhibition of ^{14}C-AMP hydrolysis by ATP exhibited anomalous kinetics: the fractional hydrolysis of AMP in the presence of ATP was higher the higher the concentration of AMP. During a 60 min incubation with the crude enzyme preparation more than 95% of the ATP was lost (as compared to incubation in buffer). About 8% of the original ATP was recovered as AMP and 3% as ADP. Thus, more than 80% of the ATP had been converted to nucleosides and bases—in all probability to a large extent via reactions catalyzed by 5′-nucleotidase. Moreover, in the presence of an excess of ATP a substantial part of the labelled monophosphate is converted to di- and triphosphate. The amount of radioactivity co-chromatographing with AMP was only 20–25% of control when ATP (100 μM) had been present during the incubation. The decrease in radioactivity found in the AMP fraction was more than compensated for by an increase in the radioactivity co-chromatographing with ADP (and/or ATP). Thus, in these experiments ATP appears to interfere with the method to determine 5′-nucleotidase acitivity by reducing the specific activity of AMP in two different ways: ATP is hydrolyzed to AMP and it converts a substantial part of the labelled AMP to labelled ADP.

Table 1. Inhibitory effect of different xanthine derivatives on soluble 5′-nucleotidase from brain. The results are expressed as IC_{20} (i.e. the dose of the xanthine that inhibits the activity by 20%)

Xanthine	IC_{20} (mM)
3-Methylxanthine	0.1
Theobromine	0.11
Theophylline	0.13
Enprofylline	0.16
Caffeine	0.17
1,7-Dimethylxanthine	0.18
1-Isobutyl-3-methylxanthine	0.30
7-Benzyl-1-isobutyl-3-methylxanthine	0.50
7-Methylxanthine	0.59
8-Phenyl-theophylline	1.82
8-*p*-Sulphophenyl-theophylline	2.09
Pentoxiphylline	4.0
HWA 285	4.3
1,3-Diethyl-8-phenyl-xanthine	>4.0
Verrophylline	>4.0

The xanthines were added in triplicates at three different concentrations. The concentration of AMP was 5 μM in these experiments. The activity of the enzyme was 65 pmol/min per mg protein.

Cytosolic 5′-nucleotidase has been purified from the bovine brain [15]. A close to 200-fold purification gave an enzyme with a V_{max} of 10–35 nKat/mg protein. In our studies of the non-purified 5′-nucleotidase from rat brain the activity was 0.1 nKat/mg protein, indicating a considerable similarity between the rat and the bovine enzyme. The K_m value determined with purified bovine brain 5′-nucleotidase [15] also agrees closely with the K_m found in rat brain. It therefore seems probable that the present way of assessing brain 5′-nucleotidase activity is adequate, despite the fact that a non-purified extract was used. The data do, however, indicate that in the absence of purification there may be problems for example when determining the effect of ATP on 5′-nucleotidase activity—at least when the present radiochemical method is used.

The results presented in Table 1 shows the potency of different xanthine derivatives as inhibitors of 5′-nucleotidase. The most potent derivatives were 3-methylxanthine, theobromine, theophylline, enprofylline and caffeine. At the concentration of 1 mM the percental inhibition obtained by these analogues were 56, 62, 43 and 45, respectively. No other analogue caused more than a 30% inhibition at this concentration. The effects of theophylline, theobromine, enprofylline and pentoxiphylline were additive to the effect of a submaximal concentration of AOPCP (3 μM), but not to the effect of a maximal (30 μM) concentration of the drug. The effect of a different set of experiments, conducted at a 4 times higher substrate concentration, are shown in Table 2. These results are essentially similar to those shown in Table 1. Figure 1 demonstrates that the xanthines cause an inhibition of the competitive type of the soluble enzyme, whereas the inhibition of the particulate enzyme appears to be non-competitive, as found before in the kidney [5].

Our results demonstrate that several xanthines are rather potent competitive inhibitors of the soluble form of 5′-nucleotidase from rat brain. The order of potency is different from the order of potency to cause inhibition of phosphodiesterase or their potency as adenosine receptor antagonists. Thus, the two 8-phenyl-substituted xanthines were the least potent of the 5′-nucleotidase inhibitors but

Table 2. The effect of xanthine derivatives on the soluble and particulate 5′-nucleotidase activity from rat brain. Results are given as enzyme activity in per cent of control at two different concentrations of the inhibitor (mean of triplicate determinations).

	Soluble		Particulate	
Xanthine*	0.1 mM	1.0 mM	0.1 mM	1.0 mM
Theobromine	89	53	74	64
3-Methylxanthine	87		94	
Theophylline	96	62	97	56
Caffeine	89	65	90	72
Enprofylline	84	75	88	84
IBMX	98	90	93	80
Pentoxiphylline	95	90	94	89
DPX	95		95	
8-Phenyl-theophylline	101	100	100	100

The activity of the enzyme was: soluble 180 pMol/min per mg; particulate 480 pMol/min per mg.

* The following abbreviations were used: IBMX for 3-isobutyl-1-methylxanthine; DPX for 1,3-diethyl-8-phenylxanthine.

are the most potent adenosine antagonists [14, 18]. The most potent nucleotidase inhibitors, 3-methylxanthine and theobromine, are among the least potent PDE inhibitors and adenosine antagonists [14]. Finally, the two 3-isobutyl-xanthines are the most potent PDE inhibitors [18, 19], but show only mediocre potency in other systems [14]. The obvious conclusion is that these three actions of the xanthines are unrelated. A corollary to this is that some xanthine derivatives may exert biological actions predominantly because they are adenosine receptor antagonists (e.g. the 8-phenyl-substituted derivatives), others may act mainly because they are phosphodiesterase inhibitors, while finally others may depend on yet other mechanisms, such as inhibition of 5′-nucleotidase, for their biological effects.

We cannot say with certainty what the biological consequences of an inhibition of soluble 5′-nucleotidase may be, but there are some interesting possibilities. Under conditions of decreased ATP synthesis or increased ATP hydrolysis AMP accumulates in a tissue. This AMP is a substrate for intracellular 5′-nucleotidase. As a consequence of its hydrolysis the adenosine concentration increases in the cell there is an efflux of adenosine. The released adenosine acts on adenosine receptors to cause different biological effects. Moreover, the total amount of cellular purines is decreased. The loss of cellular purines may lead to difficulties in restituting the intracellular ATP to initial levels upon removal of the causes for its breakdown. Hence, it is possible that a 5′-nucleotidase inhibitor may on the one hand decrease the adenosine related adaptive reactions to, for example, ischemia and hypoxia, and, on the other hand, enhance the rate of recovery after such an insult.

To summarize, the present results show that a soluble form of rat brain 5′-nucleotidase is inhibited by a series of xanthine derivatives. The most potent inhibitors were 3-methylxanthine, theobromine, theophylline, enprofylline and caffeine. The inhibition of the soluble enzyme appeared to be competitive. The structural requirements were different from that of phosphodiesterase inhibition of adenosine receptor antagonism.

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