ACTIVATION OF GUANYLATE CYCLASE BY SODIUM AZIDE IN RAT ADIPOCYTES

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1. Introduction

Since fluoride ion has been found to be a strong activator of the adenylate cyclase system [1], this same anion or another could also be a useful tool for the study of the catalytic activity of guanylate cyclase. In this regard we have therefore investigated the effect of various salts which appear to be activators of some mammalian hormonesensitive adenylate cyclase systems [2,3].

The present paper describes some kinetic properties of both the 'soluble' and the particulate guanylate cyclase* from rat adipose cells in the presence of sodium azide**. The latter a is potent activator in vitro of both enzymes, even at very low concentration. Our study shows that the activation of guanylate cyclase from rat adipocytes by sodium azide is reversible and that the progress curve displays a lag phase response.

* We have previously shown that guanylate cyclase activity was associated with a 20 000 *g* pellet and its corresponding supernatant, which were separated from an homogenate of rat adipocytes. The two forms of the enzyme have been distinguished on the basis of their dependency on the substrate, the MnGTP complex (submitted for publication).

** We have also noted a marked stimulation (25-fold) of guanylate cyclase activity by 1 mM NaN_3 in homogenates of mouse liver.

Abbreviations: Cyclic GMP, guanosine 3',5' - monophosphate; DTT, dithiothreitol.

2. Experimental procedure

Rat adipocytes [4] were homogenized in a buffer containing 1 mM DTT and 25 mM Tris-HCl, pH 7.5, as previously described [5]. Supernatant and pellet were separated after centrifugation at 20 000 *g* for 10 min. The pellet was washed twice in the original buffer, (1 mM DTT, 25 mM Tris-HCl, pH 7.5) which was used in all experiments. Protein was determined according to Lowry et al. [6]. The standard guanylate cyclase assay consisted of a regenerating system (10 mM creatine phosphate and 0.1 mg/ml creatine phosphokinase (0.5 IU/assay)), 1 mM cyclic GMP, 0.5 μCi [α^{32}P]GTP (Radiochemical Center, Amersham), 1 mM DTT, 25 mM Tris-HCl, pH 7.5 and GTP and $MnCl_2$ at the concentrations indicated in the text, in a final volume of 0.1 ml. The reaction was initiated by addition of the enzyme, and allowed to proceed for 10 min at 37°C. The reaction was stopped by dilution with 0.3 ml cold 20 mM EDTA, 50 mM Tris-HCl, pH 7.5, and 10^4 cpm [8-^{3}H]cyclic GMP were added to monitor recovery. The mixture was applied to a dry aluminium oxide column [7,8] and eluted with 3 ml 20 mM EDTA, 50 mM Tris-HCl, pH 7.5 directly onto a second column consisting of 1.25 ml Dowex 1 × 2. After washing this column with 10 ml of 0.05 N HCl, cyclic GMP was eluted with 3 ml of 0.5 N HCl.

3. Results and discussion

Since several salts, including halides and chaotropic agents, have been shown to effect adenylate cyclase activity [3], we tested their influence on the guanylate

Table 1
Effect of various salts on guanylate cyclase activity

Salt concentration		Guanylate cyclase activity (pmoles cyclic GMP·mg^{-1} protein·min^{-1}) 0.1 mM	5 mM	100 mM
None (control)	6.13			
NaF		5.85	6.42	6.03
NaCl				5.26
$NaClO_3$		6.66	7.51	2.62
$NaClO_4$		5.44	7.16	5.85
NaN_3		11.03	16.02	20.91
NaSCN		6.18	6.75	4.47
Tris·HCl				8.20

Activity was determined in an homogenate (75 μg protein) of adipocytes in the presence of 0.2 mM GTP, A mM Mn^{2+} and the various compounds at the indicated concentrations.

cyclase activity of an homogenate of rat adipocytes (table 1). When assayed at 0.2 mM GTP–1 mM Mn^{2+}, sodium salts such as fluoride, chloride, thiocyanate, chlorate and perchlorate had no noticeable effect on guanylate cyclase activity in the range 0.1–20 mM. A small degree of inhibition was observed at 100 mM for each of these compounds with the exception of Tris-HCl. In contrast, sodium azide evoked a marked increase in enzymic activity. Sodium azide (0.1 mM) stimulated 2-fold the basal activity, whereas 20 mM NaN_3 stimulated it more than 3-fold.

Since sodium azide is an inhibitor of phosphohydrolases [9,10] it was important to check that this activatory effect was not due to a sparing of the GTP substrate. The above experiment was performed under conditions in which the GTP regenerating system was highly efficient during the incubation, since 100% of the GTP added at zero time was recovered in the guanylate cyclase assay (table 2). It should be noted that only 0.1% of the GTP was transformed into [^{32}P]cyclic GMP, under standard conditions. In the absence of the regenerating system, only 20% of the total [^{32}P]GTP remained after 10 min incubation, whether or not 10 mM NaN_3 was present in the medium. Thus it appears that a high concentration of NaN_3 was unable to prevent GTP destruction by some GTPases. On the other hand, stimulation by NaN_3 could result from an inhibition of the cyclic nucleotide phosphodisterase which is present both in the soluble and particulate fractions from rat adipocytes [11,12]. This eventuality has been ruled out in experiments in which the [^{3}H]cyclic GMP (used as recovery tracer) was added either at the beginning of the incubation, or after the reaction was stopped by dilution. Effectively there was no difference in the two cases, since the same amount of [^{3}H]cyclic GMP was recovered, whether or not NaN_3 was present from 1 μM to 10 mM.

An extensive study of the kinetic properties of guanylate cyclase in the presence of sodium azide was thus undertaken. All fractions tested were sensitive

Table 2
Effect of regenerating system and NaN_3 on GTP hydrolysis in the guanylate cyclase conditions

Incubation conditions	% GTP remaining	
	$-NaN_3$	$+NaN_3$
Without regenerating system	22.3	20.6
With regenerating system	100	99.7

The homogenate (155 μg protein) was incubated for 10 min with 0.5 mM GTP (0.5 μCi [α-^{32}P]GTP), 5 mM Mn^{2+} without or with regenerating system, without or with 10 mM NaN_3, as described under Experimental Procedure. The reaction was terminated by centrifugation for 5 min at 2500 *g* and 4°C. An aliquot of each supernatant was then spotted on a PEI-cellulose plate, together with a mixture of nucleotides, and chromatographed in 1.5 M LiCl for 1 h. The R_f of GTP was 0.36. Results are expressed as the per cent radioactivity present in the GTP spot relative to the total.

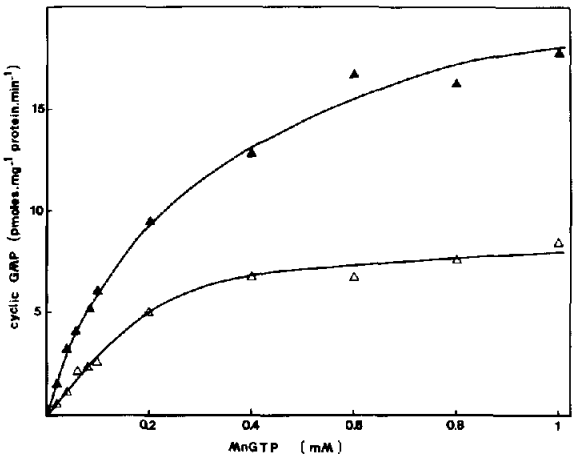

Fig.1. Guanylate cyclase activity from rat adipocytes as a function of substrate concentration. Homogenate (63 μg protein) was incubated with varying GTP concentrations and 2 mM Mn^{2+}, in the absence (△——△) or in the presence of 10 mM NaN_3 (▲——▲).

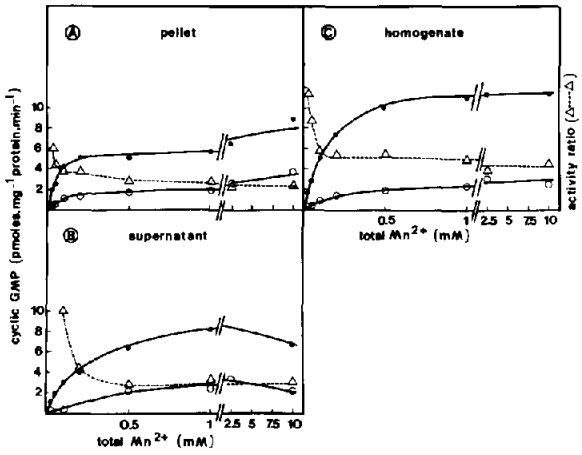

Fig.2. Effect of NaN_3 on guanylate cyclase from rat adipocytes as a function of Mn^{2+} concentration. Enzyme activity was assayed with 0.05 mM GTP, without or with 10 mM NaN_3. (A) 20 000 *g* pellet (85 μg); (B) 20 000 *g* supernatant (80 μg); (C) homogenate (78 μg protein); (○— ○) without NaN_3, (●——●) with NaN_3, (^—△) + NaN_3/−NaN_3 ratio.

to sodium azide, i.e. a crude homogenate from adipocytes and both the 20 000 *g* pellet and supernatant from the homogenate, as well as a 105 000 *g* supernatant. This activation was observed from pH 7 to pH 9.5 (data not shown).

The MnGTP-dependency of the homogenate in the presence of 10 mM NaN_3 is shown in fig.1; half-maximal activity was obtained at 0.16 mM MnGTP, whether or not NaN_3 was present; sodium azide did not therefore change the $S_{0.5}$ value for the substrate. Moreover the activation ratio (3-fold with 10 mM NaN_3) was constant over the range 0.02–1 mM GTP. This observation provided additional evidence that sodium azide did not act through GTP sparing, since in this case the enhanced activity would be strongly GTP dependent, i.e. a more marked stimulation would occur as the substrate concentration was reduced, and would become less and less as the substrate concentration increased. In contrast to the constant activation of guanylate cyclase by NaN_3 as a function of GTP, the activation was strongly dependent on the Mn^{2+} concentration. As shown in fig.2, the activity ratio in the presence or in the absence of 10 mM NaN_3 decreased when Mn^{2+} varied from 0.03 mM to 0.5 mM, and was then constant until 10 mM Mn^{2+} (GTP 0.05 mM). The presence of NaN_3 rendered the enzyme less dependent upon the concentration of free Mn^{2+}. During the course of our investigations, similar observations were recently reported in rat hepatocytes [13].

Guanylate cyclase activity was stimulated throughout a wide range of NaN_3 concentration (fig.3);

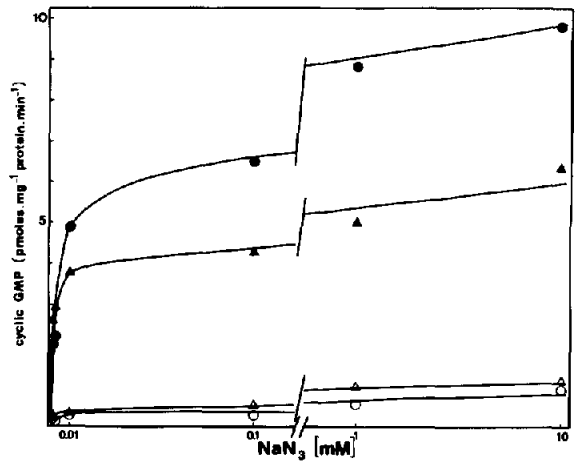

Fig.3. Guanylate cyclase activity as a function of NaN_3 concentration. Enzyme activity was assayed with 0.05 mM GTP and 0.05 mM Mn^{2+}. Homogenate (105 μg) (●——●); 20 000 *g* pellet (60 μg) (▲——▲); 20 000 *g* supernatant (122 μg) (^——△); 105 000 *g* supernatant (92 μg) (○——○).

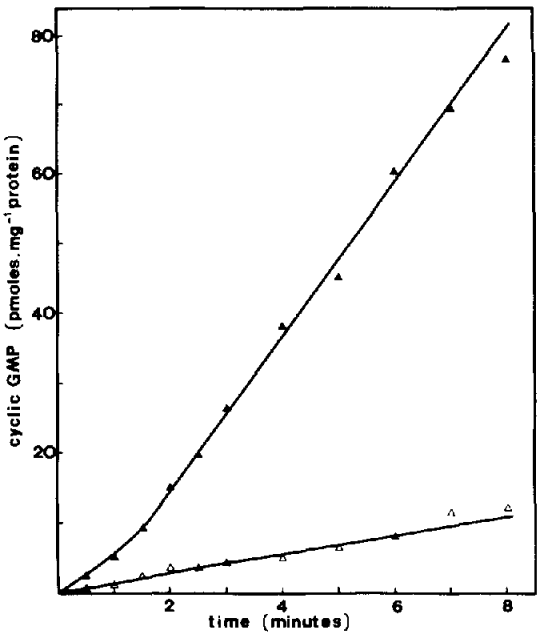

Fig.4. Effect of NaN_3 on the kinetics of guanylate cyclase activity from an homogenate. Protein (63 μg) was incubated with 0.05 mM GTP and 2 mM Mn^{2+} in the absence (△——△) or in the presence of 10 mM NaN_3 (▲——▲) in a final volume of 50 μl.

pellet and homogenate enzymes exhibited a marked stimulation at 1 μM NaN_3, whereas 'soluble' fractions were less sensitive, since activation was noticeable only from 0.1 mM NaN_3.

When the homogenate of adipocytes was assayed in the presence of 10 mM NaN_3, the activation phenomenon was evident at 15 sec but presented a lag phase of about 45 sec, after which the reaction rate was constant (fig.4). We similarly observed such a lag phase in the kinetics of adenylate cyclase activity in plasma membranes from rat adipocytes when assayed in the presence of sodium fluoride (unpublished results).

On the other hand, the reversibility of the activation by NaN_3 was tested by preincubating the particulate enzyme (20 000 *g* pellet) without or with NaN_3 and then washing them extensively (table 3). The basal activity of the enzyme preincubated with 10 mM NaN_3 was not different from the basal activity of the control enzyme (preincubated without NaN_3). Activation after readdition of 10 mM NaN_3 was

Table 3
Reversibility of the effect of sodium azide

Treatment	Guanylate cyclase (pmoles·mg^{-1} protein·min^{-1}) Basal	+10 mM NaN_3
Initial activity	0.15	0.28
Preincubation:		
Control	0.12	0.32
NaN_3	0.13	0.25

Protein (120 μg) (20 000 *g* pellet) were incubated without or with 10 mM NaN_3 in the presence of 0.05 mM GTP, 0.2 mM Mn^{2+}, 1 mM cyclic GMP, 1 mM DTT, 25 mM Tris-HCl pH 7.5 in a final volume of 0.9 ml for 10 min at 37°C. The reaction was terminated by a 10-fold dilution with 1 mM DTT, 25 mM Tris-HCl, pH 7.5, and then centrifugated 10 min at 20 000 *g*. After a second identical washing the pellet was resuspended in 0.4 ml 1 mM DTT, 25 mM Tris-HCl, pH 7.5 and 50 μl-aliquots were assayed for guanylate cyclase activity, with 0.05 mM GTP and 0.2 mM Mn^{2+}.

almost entirely recovered (table 3); activation was therefore reversible. For this purpose we have to note that reversion of activation of hepatic adenylate cyclase by sodium azide and sodium fluoride has been recently observed after extensive washing of liver membranes [3]. In contrast, the conclusion that activation of the hepatic guanylate cyclase by NaN_3 was irreversible was reached on the basis of dilution and dialysis of the reaction mixtures [13]. The reversibility of the activation by NaN_3 of rat adipocyte guanylate cyclase, considered together with the delayed response of the enzyme to the activatory anion, may reflect induced conformational changes rather than direct activation of the catalytic site. The time dependent changes in enzyme activity seen in response to regulatory ligands have been discussed in detail [14,15]; they may indicate isomerization of (de)polymerisation processes. Additional studies will be necessary to relate our present observations with an eventual oligomeric structure of the guanylate cyclase.

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