Guanine nucleotides and adenosine 'R$_i$'-site analogues stimulate the membrane-bound low-K_m cyclic AMP phosphodiesterase of rat adipocytes

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Concentrations of GTP or Gpp(NH)p up to 300 nM activated the membrane-bound low-K_m cyclic AMP phosphodiesterase of rat adipocytes, while higher concentrations of these nucleotides reversed this activation. The adenosine analog N^6-phenylisopropyladenosine (N^6-PIA) elicited a dose-dependent stimulation of this enzyme (K_{act} = 3 nM), an effect which did not require GTP and which was additive with the GTP-induced stimulation. Both the N^6-PIA and GTP stimulations were rapid, reversible and resulted from an increase in V_{max}. In contrast, neither GTP, nor N^6-PIA affected the soluble low-K_m cyclic AMP phosphodiesterase.

Guanine nucleotide *Adenosine analog* *Phosphodiesterase* *Adipocyte* *Rat*

1. INTRODUCTION

Previous data [1] from this laboratory have shown that the particulate low-K_m cyclic AMP phosphodiesterase from rat brain is stimulated by low concentrations of GTP. In the same study, the adenosine analog N^6-phenylisopropyladenosine (N^6-PIA) was found to stimulate this enzyme as well, provided that high concentrations of GTP were present in the assay. It was thus proposed that a guanine-nucleotide regulatory protein could be involved in the molecular mechanism of phosphodiesterase stimulation induced by both GTP and N^6-PIA.

Recently, it was reported that following incubation of isolated fat cells with adenosine, the whole phosphodiesterase activity tested in adipocyte homogenates was increased [2]. Since great similarities in the adenosine actions exist between the brain and the fat cell, our purpose was to determine whether the model proposed for the membrane-bound low-K_m cyclic AMP phosphodiesterase of brain could also apply to the adipocyte.

2. MATERIALS AND METHODS

Cyclic [^{3}H]AMP was from Amersham, collagenase (CLS) from Worthington, N^6-phenylisopropyladenosine, adenosine deaminase, GTP and Gpp(NH)p from Boehringer, and bovine serum albumin (fatty acid-poor) from IBF.

Male Wistar rats (CERJ) weighing 150–220 g were decapitated, and epididymal fat pads quickly removed. Isolated fat cells were prepared by a slight modification (reduction of the collagenase digestion period to 30 min) of the procedure described previously [3]. Crude membranes were prepared as in [4] with slight modifications: isolated fat cells were washed 3 times with Krebs-Ringer bicarbonate buffer (pH 7.4) and suspended in 250 mM sucrose/1 mM EDTA/10 mM Tris–HCl buffer (pH 7.4). After mechanical disruption, the resulting broken fat cell suspension was centrifuged at 20000 × g for 15 min. The final

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pellet was washed with 10 mM $MgCl_2$/50 mM Tris–HCl (pH 7.4) and resuspended in 1 mM $KHCO_3$, resulting in a suspension containing 2–4 mg protein/ml.

Determination of the low-K_m cyclic AMP phosphodiesterase activity was performed following the methods described in [5] and [6] with slight modifications. Briefly, incubations were performed in a final volume of 250 μl containing 0.02 μM cyclic [^{3}H]AMP, 0.5 μM cyclic AMP, 30 mM Tris–HCl buffer (pH 7.4), 5 mM $MgCl_2$, 0.04% bovine serum albumin, 0.5 unit/ml adenosine deaminase and, when indicated, various concentrations of GTP, Gpp(NH)p or N^6-PIA. Reactions were initiated by the introduction of 15–40 μg protein and, after 5 to 10 min of incubation at 37°C, incubations were stopped by boiling the tubes for 90 s. After precipitation of 5'-AMP by addition of 200 μl $Ba(OH)_2$ (0.3 N) and 200 μl $ZnSO_4$ (0.3 M), each tube was mixed and centrifuged (5000 × g for 10 min). Aliquots of the supernatant were counted, and compared to values obtained from tubes boiled without incubation. Conversion of cyclic AMP into products was not allowed to exceed 15% over the incubation period time (5–10 min at 37°C) and was linear during this time and with the protein concentration (up to 50 μg protein). Protein was determined according to [7].

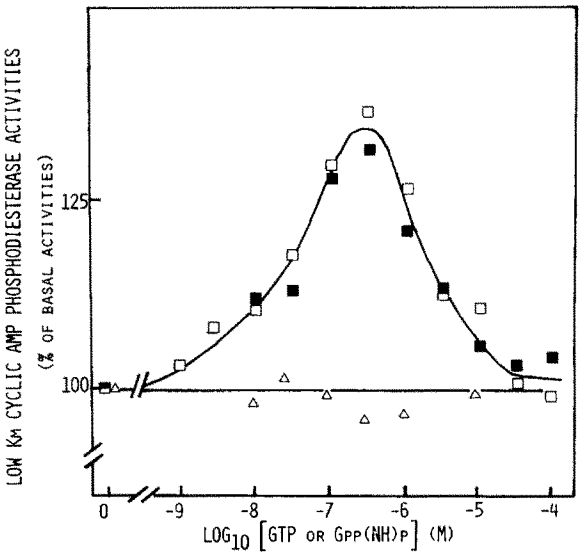

Fig.1. Dose-response curve to GTP (□, △) and Gpp(NH)p (■) of the membrane-bound (□, ■) and soluble (△) low-K_m cyclic AMP phosphodiesterase activities of rat white adipocyte. Data are expressed as percentage over basal activity and represent the mean values of 3–5 experiments performed in triplicate. Triplicate differed by less than 5%. Mean ± SE basal activities were 32 ± 4 pmol·mg^{-1}·min^{-1} for the membrane-bound (n = 8) and 20 ± 4 pmol·mg^{-1}·min^{-1} for the soluble phosphodiesterase (n = 4).

3. RESULTS AND DISCUSSION

As shown in fig.1, the particulate low-K_m cyclic AMP phosphodiesterase, contrary to the soluble enzyme which was insensitive, elicited a biphasic response to GTP with, at concentrations up to 300 nM, an activation (maximal stimulation +35 ± 9% over basal activity, apparent K_{act} = 43 ± 9 nM, n = 5) and, at higher concentrations, progressive reversal of this activation (the activity is brought back to the basal activity at 30 μM GTP).

Kinetic studies revealed that the time-course of this effect was rapid (equilibrium being reached at the second minute of incubation) and persistent (for at least 13 min, not shown). Furthermore, this effect was reversible: experiments in which the particulate fraction was preincubated for 5 min without substrate and in the absence (control) or presence of 1 μM GTP, washed and then tested for phosphodiesterase activity, showed no more difference between the control and the GTP-exposed fractions (not shown).

Linear regression analysis of the double-reciprocal plots of cyclic AMP hydrolysis performed in the 80 nM–5 μM cyclic AMP concentration range (to avoid interference from the high-K_m phosphodiesterase) and in the absence or presence of 300 nM GTP (fig.2), revealed that the stimulatory effect of GTP resulted from an increase in V_{max} rather than from a change in K_m (0.97 μM *vs* 0.72 μM in control).

Phosphodiesterase activation induced by low GTP concentrations could not be due to cyclic GMP generated from GTP by guanylate cyclase, since, contrary to the observations made in other tissues [8,9], cyclic GMP was recently shown to be an inhibitor of the particulate low-K_m cyclic AMP phosphodiesterase of differentiated adipocyte [10]. Furthermore, Gpp(NH)p, a non-hydrolysable analog of GTP, elicited the same effects as GTP on phosphodiesterase: in fact both dose-response

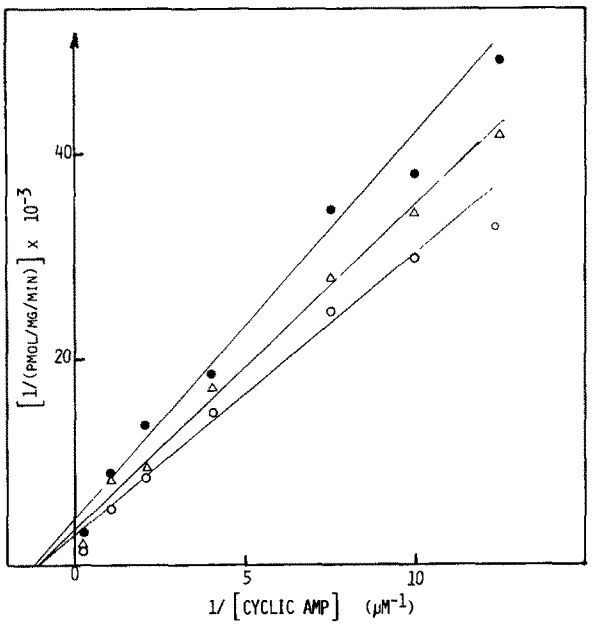

Fig.2. Lineweaver-Burk plot of stimulation of the adipocyte membrane-bound low-K_m cyclic AMP phosphodiesterase induced by GTP or N^6-phenylisopropyladenosine (N^6-PIA). Membrane-bound low-K_m cyclic AMP phosphodiesterase activities were determined as described in section 2, in the absence (●) and presence of 300 nM GTP (○), or 10 nM N^6-PIA (△). Each point is the mean of quadruplicate determinations from one experiment. K_m values were 0.73, 0.97 and 0.72 μM in the absence and presence of GTP or N^6-PIA, respectively. V_{max} values were 207, 384 and 280 pmol·mg^{-1}·min^{-1} in the absence and presence of GTP or N^6-PIA, respectively.

curves were strictly superimposable (fig.1). This finding indicates that both the activatory and deactivatory effects of GTP on phosphodiesterase do not require GTP hydrolysis, and, because of the absence of ATP in the assays, do not involve a phosphorylation-dependent mechanism. Moreover, these data strongly suggest the existence of two different GTP-dependent regulatory sites for the membrane-bound phosphodiesterase, one displaying high affinity for GTP and mediating activation, and the other having lower affinity and inhibiting this enzyme.

Because a recent report has shown that an exposure of adipocytes to pertussis toxin results in a marked inhibition of the ability of insulin to stimulate the membrane-bound phosphodiesterase [11], it is tempting to speculate that the GTP-dependent regulatory sites postulated above might be identical with or similar to the N_i and N_s proteins involved in the adenylate cyclase-coupling processes (reviewed in [12]). As already mentioned, only the particulate low-K_m cyclic AMP phosphodiesterase is stimulated by insulin in mature adipocyte [13]. In these cells, it is now well established that adenosine exerts some insulin-like effects [14]. As guanine-nucleotides play a role in the regulation of phosphodiesterase in brain cortex [1], retina [15,16] and hepatocytes [17] at least, and since the R_i-site adenosine-agonist N^6-PIA exerts a GTP-dependent modulation of the particulate phosphodiesterase of rat brain [1], the possibility was investigated that in adipocytes this enzyme might be modulated by N^6-PIA as well.

As shown in fig.3, while the soluble enzyme was insensitive to N^6-PIA (whether GTP was present or not), the membrane-bound enzyme elicited an activation in response to N^6-PIA. This effect occurred at extremely low N^6-PIA concentrations (apparent K_{act} = 3 ± 0.8 nM), maximal stimulation (+30 ± 2% over basal value) being reached at 10 nM. Like the GTP-activatory effect described above, the time-course of the N^6-PIA stimulatory effect was rapid (equilibrium reached in less than 2 min), persistent (for 13 min, at least) and reversible (data not shown). Moreover, linear-regression

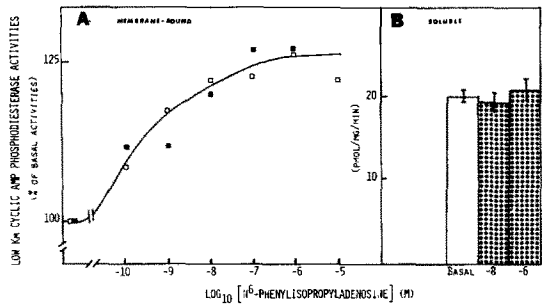

Fig.3. Influence of N^6-phenylisopropyladenosine (N^6-PIA) on the membrane-bound and soluble low-K_m cyclic AMP phosphodiesterase activities of rat adipocytes. (A) Dose-response curves to N^6-PIA of the membrane-bound enzyme studied in the absence (□) or presence of 100 nM GTP (■). Data are expressed as percentage over the corresponding basal values (absence or presence of GTP) and are the mean values of 4 separate experiments performed in triplicate. (B) Effects of N^6-PIA (10 nM and 1 μM) on the soluble enzyme. Each value is the mean ± SE of 4 separate experiments performed in triplicate.

analysis of the double reciprocal plots of cyclic AMP hydrolysis (assayed in the presence of 10 nM N^6-PIA) showed no effect on the K_m value (0.72 μM *vs* 0.73 μM in control) but an increase in the V_{max} value (fig.2). Dose-response curves to N^6-PIA (expressed as % activation over basal value) resulted in strictly superimposable curves whether these experiments were performed in the absence or presence of maximal stimulatory concentrations of GTP (100 nM) in the assay. This finding, which indicates that both the GTP and the N^6-PIA effects are additive, suggests that these two effects are mediated by different mechanisms.

In brain, where both 'R_a' and 'R_i' sites coexist (review in [18]), we found that N^6-PIA elicited a biphasic effect on the particulate low-K_m cyclic AMP phosphodiesterase. In the adipocyte, where only 'R_i' sites seem to be present [19], this study shows that this adenosine-analog induces only a stimulation of this enzyme. It is thus tempting to postulate that this effect is mediated through the adenosine 'R_i' binding sites. However, the mechanisms involved in the N^6-PIA induced phosphodiesterase stimulation are probably different from those implicated in the 'R_i'-site-mediated inhibition of adenylate cyclase. In fact, in the adipocyte, GTP is required for the adenylate cyclase inhibition [20] but not, as shown here, for the phosphodiesterase stimulation, whereas in the brain, GTP appears necessary for both effects [1,21].

Finally, the present report may offer a possible explanation for the fact that maximal adenylate cyclase inhibition found with N^6-PIA in adipocyte membranes under the usual assay conditions (i.e., when cyclic AMP breakdown is prevented) never exceeds 50% [22], while with the same N^6-PIA concentrations, the inhibition of lipolysis found in intact cells is almost 100% [22,23].

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