Guanine nucleotide- and GTP-dependent N^6-phenylisopropyladenosine stimulation of the membrane-bound cyclic AMP high affinity phosphodiesterase in rat brain

Philippe de Mazancourt and Yves Giudicelli*

Service de Biochimie de la Faculté de Médecine de Paris-Ouest et du Centre Hospitalier de Poissy, 78303 Poissy Cédex, France

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In brain cortex, low concentrations of GTP or Gpp(NH)p activated the membrane-bound low K_m cyclic AMP phosphodiesterase while higher concentrations of GTP, but not of Gpp(NH)p, reversed this activation. The adenosine analog N^6-phenylisopropyladenosine (N^6-PIA) elicited biphasic effect on this enzyme (activation up to 10^{-8} M, complete reversion at 10^{-5} M), provided that GTP was present. N^6-PIA activation was reduced in the presence of Gpp(NH)p and blocked by sodium (80 mM). In contrast, the soluble low K_m cyclic AMP phosphodiesterase was insensitive to GTP or N^6-PIA. This study suggests that guanine nucleotides and N^6-PIA exert their effects on the membrane-bound enzyme through guanine nucleotide regulatory proteins.

Membrane-bound phosphodiesterase *Guanine nucleotide* *Adenosine analog* *Guanine nucleotide regulatory protein* *Brain cortex*

1. INTRODUCTION

Previous studies from this laboratory [1] have shown that adrenalectomy results in an impaired reactivity of the adenylate cyclase–phosphodiesterase system in response to the 'R' adenosine site agonist, N^6-phenylisopropyladenosine (N^6-PIA), in rat white fat cells. In normal brain, a tissue sharing common properties with the adipocyte, at least with regard to the coexistence of 'Ri' and 'Ra' adenosine sites (review [2]), we have recently provided evidence suggesting that in the presence of the guanine nucleotide GTP, N^6-PIA may stimulate the low K_m cyclic AMP phosphodiesterase [3]. Furthermore, in the same study we also found that N^6-PIA inhibited this enzyme after adrenalectomy.

The aim of the present work was to clarify the role played by GTP in both the regulation of the brain low K_m cyclic AMP phosphodiesterase and in the effects of N^6-PIA on this enzyme.

Furthermore, as low K_m cyclic AMP phosphodiesterase activity has been detected in both the membranes and the cytosol of several tissues [4,5] including the brain [6], we have focused this study on these two forms of this enzyme.

2. MATERIALS AND METHODS

[^{3}H]Cyclic AMP (36 Ci/mmol) was from the Radiochemical Centre Amersham, N^6-PIA, adenosine deaminase, cyclic AMP, Gpp(NH)p and GTP from Boehringer. The phosphodiesterase inhibitor Ro-1724 was kindly supplied by Hoffman-La Roche.

Brain cortical membrane and cytosolic fractions were prepared from Wistar male rats (150 g) as follows. Cortex from freshly decapitated rats were sliced and incubated at 37°C for 30 min in Krebs-Ringer phosphate buffer. The brain slices were

* To whom correspondence should be addressed

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then transferred to ice-cold Tris–HCl buffer (50 mM, pH 7.4), homogenized and centrifuged at 800 × *g* for 5 min at 4°C. The pellet was discarded and the supernatant centrifuged at 3000 × *g* for 10 min at 4°C. The resulting supernatant and pellet were separated and recentrifuged at 48000 × *g* for 30 min at 4°C. The pellet issued from the centrifugation of the 3000 × *g* pellet was used to investigate the 'membrane-bound' low K_m cyclic AMP phosphodiesterase activity, while the supernatant issued from the centrifugation of the 3000 × *g* supernatant served as the source of the soluble form of this enzymic activity.

The low K_m cyclic AMP phosphodiesterase activity was assayed by a slight modification of the method in [3]. Incubations were performed in a final volume of 600 μl containing 0.02 μM [^{3}H]cyclic AMP, 0.08 μM cyclic AMP, 30 mM Tris–HCl buffer (pH 7.4), 5 mM $MgCl_2$, 0.04% bovine serum albumin, 0.25 units/ml adenosine deaminase and, when indicated, GTP or Gpp(NH)p at various concentrations. Reactions were initiated by the introduction of 20–50 μg protein and, after 5 min of incubation at 25°C, reactions were stopped by boiling the tubes for 90 s after which low K_m cyclic AMP phosphodiesterase activities were determined as in [3]. Conversion of cyclic AMP into products was not allowed to exceed 15% over the incubation period time (5 min at 25°C) and was linear during this time and with protein (up to 100 μg protein).

3. RESULTS AND DISCUSSION

To avoid the interference of the high K_m phosphodiesterase activities, kinetic studies of the soluble and membrane-bound low K_m phosphodiesterase activities were first performed with substrate concentrations ranging from 0.1 to 5 μM. Under these conditions, the K_m values found for the soluble and membrane-bound enzymes were 1.5 ± 0.1 and 1.0 ± 0.2 μM (n = 3), respectively, which are in good agreement with those reported in [7].

From fig.1, it is clear that the soluble phosphodiesterase is insensitive to the addition of GTP in the incubation medium. On the contrary, the membrane-bound activity was sensitive to GTP, showing a biphasic response with, at GTP concentrations up to 30 nM, an activation and, at higher concentrations, a progressive reversal of this activation (the activity is brought back to the basal level at 1 μM GTP). Kinetic studies revealed that the time-course of the GTP (30 nM) activatory effect was rapid, equilibrium being reached within the first min of incubation (not shown). Furthermore, this effect was reversible. In fact, experiments in which the membrane-bound fraction was preincubated in the substrate-free phosphodiesterase assay medium without (control) or with 30 nM GTP for 5 min, washed twice and then tested for phosphodiesterase activity, showed no difference between the control and the GTP-exposed fractions (not shown).

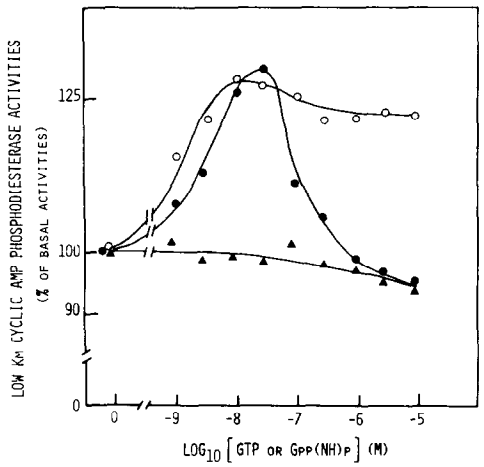

Fig.1. Dose–response curves to GTP and Gpp(NH)p of the membrane-bound and soluble low K_m cyclic AMP phosphodiesterase in rat brain cortex. Data are expressed as percentage over basal activity and represent the mean values of 3–5 separate experiments performed in triplicate which differed by less than 5%. (●) Membrane-bound phosphodiesterase with GTP, (○) membrane-bound phosphodiesterase with Gpp(NH)p, (▲) soluble phosphodiesterase with GTP. Mean ± SE control activities (no addition) were 57 ± 5 pmol·mg^{-1}·min^{-1} for the membrane-bound (n = 8) and 115 ± 17 pmol·mg^{-1}·min^{-1} for the soluble phosphodiesterase (n = 4).

Since cyclic GMP has been shown to be an activator of the phosphodiesterase particulate form [8], it could not be excluded that the activation by GTP of the membrane-bound enzyme could be artifactually due to the presence of cyclic GMP generated from GTP by guanylate cyclase. This possibility seems, however, unlikely for the follow-

ing reasons. First, half-maximal activation of the phosphodiesterase by cyclic GMP occurs in the 10 μM range [8] which is at least 3 orders of magnitude higher than the GTP concentrations eliciting half-maximal activation of the enzyme. Second, as seen in fig.1, the non-hydrolysable GTP analog, Gpp(NH)p, also activated the membrane-bound phosphodiesterase, a result which is uncompatible with a cyclic GMP-dependent process and which furthermore rules out the possibility that a direct phosphorylation of the enzyme by GTP may be involved in this process. Although low concentrations of both GTP and Gpp(NH)p activated the membrane-bound phosphodiesterase, concentrations of Gpp(NH)p above 10^{-7} M did not lead, contrary to GTP, to a reversion of the activation process (fig.1). This suggests that GTP hydrolysis is probably required for the expression of the GTP inhibitory process.

The bimodal action of GTP on the membrane-bound phosphodiesterase reported here is intriguing considering the analogies existing between this action and the well established bimodal regulation of adenylate cyclase systems by guanine nucleotides (review [9]). In fact the data in fig.1 are compatible with the existence of two different GTP-dependent regulatory sites for phosphodiesterase, one displaying high affinity for GTP and mediating activation of the membrane-bound enzyme and the other one having lower affinity and inhibiting the enzyme only when GTP is hydrolyzed. It is thus tempting to postulate that the membrane-bound phosphodiesterase is positively and negatively coupled to two different guanine-nucleotide regulatory proteins resembling the 'Ni' and 'Ns' proteins involved in adenylate cyclase coupling processes [9].

To test this hypothesis, the possibility was investigated that the membrane-bound phosphodiesterase might be biphasically modulated by the adenosine analog N^6-PIA which, through binding to Ri or Ra sites, exerts a GTP-dependent adenylate cyclase inhibition or activation (review [2]). As shown in fig.2, while the soluble enzyme was insensitive to N^6-PIA (whether GTP was present or not), the membrane-bound enzyme elicited a biphasic response to N^6-PIA provided that high concentrations (10^{-5} M) of GTP were present in the assay. Under these conditions, extremely low N^6-PIA concentrations (up to 0.3 nM) stimulated

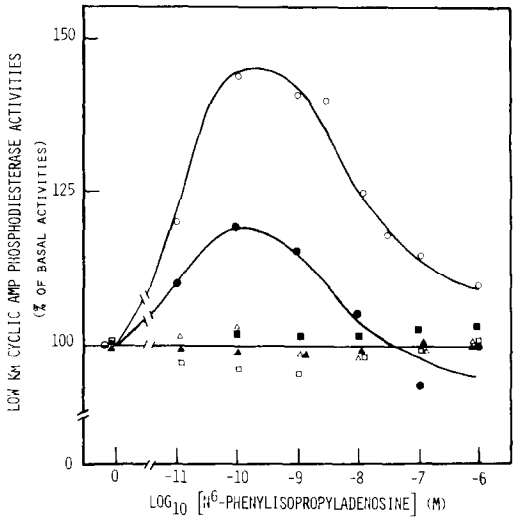

Fig.2. Dose–response curves to N^6-phenylisopropyladenosine of the membrane-bound and soluble low K_m cyclic AMP phosphodiesterase in rat brain cortex. Values are expressed as in fig.2 and are the mean values of 3–5 separate experiments performed in triplicate. (▲) Membrane-bound enzyme without guanine nucleotides, (□) membrane-bound enzyme with GTP (10^{-8} M), (○) membrane-bound enzyme with GTP (10^{-5} M), (●) membrane-bound enzyme with Gpp(NH)p (10^{-5} M), (■) membrane-bound enzyme with GTP (10^{-5} M) and Na^+ (80 mM), (△) soluble enzyme with GTP (10^{-5} M).

the enzyme (EC_{50} = 0.015 nM, maximal effect +45%), whereas higher concentrations tended to reverse this response (EC_{50} = 15 nM). Like the GTP-activatory effect described above, the time-course of the phosphodiesterase stimulation induced by 0.3 nM N^6-PIA was rapid (equilibrium being reached in less than 1 min) and was reversible (not shown).

Surprisingly, addition of 80 mM sodium abolished the activatory and inhibitory effects of N^6-PIA (fig.2). As already mentioned, these two effects were strictly dependent on the presence of high GTP concentrations, since, as shown in fig.2, the membrane-bound enzyme became unresponsive to N^6-PIA when studied in the absence or in the presence of 10^{-8} M GTP. As also shown, degrees of activation and inhibition by N^6-PIA were half-reduced when GTP was replaced by 10^{-5} M Gpp(NH)p. As this unexpected finding was observed at all N^6-PIA concentrations tested, it cannot be explained by the reduced binding af-

finity of the Ri sites for adenosine agonists which is induced by Gpp(NH)p [10]. Moreover, this finding also excludes the possibility that the phosphodiesterase activation due to N^6-PIA involves either a GTP-mediated phosphorylation of the enzyme or, because these experiments were performed in the absence of ATP, enzyme phosphorylation mediated by ATP. Thus, the mechanism through which N^6-PIA modulates the membrane phosphodiesterase in the brain appears different from the ATP-dependent phosphorylation mechanism which has been recently proposed [11] to explain the insulin-stimulation of phosphodiesterase in liver plasma membranes.

From these results, there are some arguments suggesting that both the activatory and inhibitory effects of N^6-PIA on the membrane-bound phosphodiesterase might be mediated through the adenylate cyclase negatively and positively coupled Ri and Ra sites, respectively. In fact:

(i) the time-course of occurrence of these two effects was rapid (in the min range);
(ii) the N^6-PIA concentrations eliciting phosphodiesterase activation and inhibition were compatible with the N^6-PIA concentrations required to observe N^6-PIA binding to the Ri and Ra sites respectively [2];
(iii) like the Ri and Ra site-mediated effects of N^6-PIA on adenylate cyclase [9], activation and inhibition of phosphodiesterase by N^6-PIA required GTP;
(iv) finally, only the membrane-bound enzyme was found sensitive to both effects of N^6-PIA.

Whether the GTP-dependent N^6-PIA effects on phosphodiesterase are mediated or not by GTP-binding proteins remains an open question. Supporting such a mediation are several recent reports [12–14] concerning other GTP-dependent membrane-bound phosphodiesterases: the insulin-stimulated cyclic AMP phosphodiesterase of the liver [9] or the photolyzed activated cyclic GMP phosphodiesterase of the rod outer segments [13–16] which depends on transducin, a GTP-dependent protein structurally similar [17] but functionally different from the Ni and Ns proteins involved in adenylate cyclase regulation [18–20]. The same situation may also apply to the N^6-PIA effects on the membrane-bound cyclic AMP phosphodiesterase of the brain since:

(i) Gpp(NH)p which prevents the Ni-mediated inhibitory effect of N^6-PIA on adenylate cyclase [12], reduced but did not abolish the N^6-PIA-induced phosphodiesterase stimulation;
(ii) sodium ions, which promote the Ni-mediated inhibition of adenylate cyclase by N^6-PIA [18] without affecting the Ns-mediated adenylate cyclase activation [9], suppressed both the N^6-PIA-induced phosphodiesterase activation and inhibition.

Further studies using toxins which specifically alter Ns [19] or Ni [20–22] would be helpful to elucidate the nature of the factors mediating the N^6-PIA effects reported here.

Finally, this study suggests an important role for adenosine in the control of cyclic AMP metabolism in the brain cortex. In fact, N^6-PIA, at least at low concentrations, contributes to decrease cyclic AMP levels not only because it is a well known inhibitor of adenylate cyclase [23], but also, as shown here, because it stimulates cyclic AMP phosphodiesterase. As the latter effect is also induced by insulin [5–24], the presently reported phosphodiesterase stimulation by N^6-PIA constitutes an additional example of an insulin-like action of adenosine [25].

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