Multiple Controls by Adenosine Receptors on the Adenylate Cyclase in the Rat Hepatic Membrane

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Abstract—The effects of an adenosine analog, N⁶-phenyl-isopropyl-adenosine (PIA), on the glucagon-stimulated adenylate cyclase activity in rat hepatic membranes were studied. Adenosine at high concentrations (>10 μ M) has been reported exclusively to inhibit the adenylate cyclase via intracellular P-sites of the hepatic membrane. The stimulation by glucagon of the enzyme was attenuated by nanomolar concentrations of PIA in the presence of low concentrations (<1.0 μ M) of GTP, indicating the effect of the guanine nucleotide inhibitory system (Ni). This inhibition by PIA required the presence of sodium chloride and was antagonized with isobutyl methylxanthine, an antagonist for the extracellular R-site receptors. The inhibitory effects of PIA disappeared and reversed into a stimulatory phase with increasing concentrations of GTP, suggesting the presence of a stimulatory (Ns) and an inhibitory (Ni) guanine nucleotide system of the enzyme in the action of the adenosine. PIA concentrations over a micromolar were observed to stimulate the enzyme activity in a GTP-dependent manner, indicating the presence of the stimulatory receptor (A₂ or Ra) coupled to the Ns. These results suggest that receptors for adenosine of the inhibitory type (A₁ or Ri) and the stimulatory type (A₂ or Ra) are present on the rat hepatic membrane, showing multiple controls of the adenylate cyclase system, depending on the cellular concentrations of GTP and/or sodium chloride.

Adenosine has been reported to regulate adenylate cyclase with stimulatory and/or inhibitory effects in many tissue systems (1). The effects of nanomolar concentrations of adenosine are reported to be mediated through extra-cellular R-sites requiring integrity of the ribose ring, but modifications on the purine ring subdivided the receptor sites into stimulatory (A₂ or Ra) and inhibitory ones (A₁ or Ri) (2, 3). The effects of higher than micromolar concentrations of adenosine appear to be mediated via intracellular P-sites with an intact purine moiety, but modification of the ribose moiety of the molecule caused it to be inhibitory towards the enzyme (1, 4). Studies on adrenal tumor cells (5, 6) and bone cells (7) indicate that relatively high concentrations (>10 μ M) of adenosine and related nucleosides stimulate adenylate cyclase activity. On the contrary, Glynn and

Cooper (8) and Shima (9) have reported an inhibitory action of adenosine on the ACTH-stimulated adenylate cyclase activity of adrenocortical membranes.

In hepatic membranes, adenosine has been reported exclusively to inhibit the adenylate cyclase system (10, 11). This inhibition, however, was apparent in the presence of relatively high concentration (>10 μ M) of adenosine (10, 11), indicating that the effect was mediated via intracellular P sites (1, 4).

On the contrary, stimulation of the adenylate cyclase-cyclic AMP system by adenosine agonists for extracellular R-sites has been observed in the hepatic membrane (3, 12–14).

The purpose of this investigation was to examine the precise mechanism of these dual effects of adenosine on extracellular R-sites of the adenylate cyclase moiety in the rat

hepatic membrane.

Materials and Methods

Cyclic AMP was generously donated by Daiichi Seiyaku, Tokyo, Japan. Glucagon was obtained from Calbiochim-Hoechst, Tokyo, Japan. (+)-N⁶-phenylisopropyl-adenosine (PIA) and 3-methyl-1-isobutylxanthine (IBMX) were from Boehringer-Mannheim, F.R.G. and Aldrich Chemical Co., Milwaukee, WI, respectively.

Preparation of hepatic membranes: Male rats of the Donryu strain (Nippon Rats Co., Ltd., Tokyo, Japan), weighing between 180 and 200 g, were used. After the liver had been perfused to remove blood, pieces of approx. 2.0 g were removed to the homogenization medium containing 154 mM NaCl, 20 mM sodium-Hepes (pH 7.5). Plasma membranes obtained from the procedure of Neville (15) up to step 11 were suspended in 10 volumes of 10 mM sodium-Hepes (pH 7.5), 1 mM mercapto-ethanol, centrifuged at 24,000×g for 10 min at 4°C, washed three times, and dialyzed for 2 hr in the cold buffer containing 0.01% activated charcoal to remove endogenous adenosine associated with the membranes. Membrane protein was determined by the method of Lowry et al. (16).

Adenylate cyclase assays: Adenylate cyclase activity was estimated by incubation of the membranes in 40 mM Tris-HCl buffer (pH 7.4), containing 4 mM MgCl₂, 5 mM theophylline, 2 mM ATP, 100 µg/ml pyruvate kinase, and 5 mM phosphoenolpyruvate for 10 min, as previously described (17–19). Cyclic AMP formed during incubation was estimated by a competitive binding assay (20), using a protein purified from rabbit skeletal muscle through the DEAE-cellulose column step.

Results

Hepatic adenylate cyclase activity from male adult rats was responsive to increasing concentrations of glucagon in the presence of 1 µM GTP (Fig. 1). As shown in Fig. 2, stimulation by glucagon of adenylate cyclase activity was GTP-dependent. Figs. 3 and 4 present the activation of adenylate cyclase by PIA with regard to GTP-dependency. The concentration of PIA required for half-maxi-

Fig. 1. Effects of glucagon on hepatic adenylate cyclase activity in the presence of 1 µM GTP. Adenylate cyclase was assayed as described under Materials and Methods. The incubation with various concentrations of glucagon was carried out for 10 min. Results shown are the means±S.E. of four incubations.

mal stimulation (ED₅₀) was approximately 1.0 µM (Fig. 3). Concentrations of GTP required for half-maximal stimulation (ED₅₀) were in the range of 0.1 µM (Fig. 4). The stimulation by 10 nM glucagon of adenylate cyclase activity was attenuated by PIA in a dose-dependent manner (Fig. 5). The concentration resulting in half-maximum inhibition (IC₅₀) of PIA was in the range of nanomolar concentrations. This inhibition by PIA absolutely required the presence of 200 mM sodium chloride.

In the absence of added GTP, there was no inhibition of the glucagon-stimulated enzyme by PIA (Fig. 6). Figure 6 demonstrates the GTP-dependent process for the inhibitory action of PIA on the glucagon-stimulated activity at micromolar concentrations of the

nucleotide. GTP over $10 \mu\text{M}$ exclusively manifested a stimulation by glucagon (Fig. 6).

Fig. 2. GTP-dependent stimulation of glucagon-activated adenylate cyclase activity in hepatic membranes. Membranes were incubated with increasing concentrations of GTP in the presence of 10 nM glucagon. Means $\pm$ S.E. of four incubations are shown.

Fig. 3. Effects of N^6 -phenylisopropyladenosine (PIA) on adenylate cyclase activity in hepatic membranes. Adenylate cyclase activity in the presence of $1 \mu\text{M}$ GTP was assayed with increasing concentrations of PIA. Results shown are the means $\pm$ S.E. of triplicate incubations.

Fig. 4. GTP-dependent stimulation of N^6 -phenylisopropyladenosine (PIA)-activated adenylate cyclase activity in hepatic membranes. Membranes were incubated with increasing concentrations of GTP in the presence of $10 \mu\text{M}$ PIA. Means $\pm$ S.E. of triplicate incubations are shown.

Fig. 5. Effects of sodium chloride on inhibition of glucagon-stimulated adenylate cyclase activity by N^6 -phenylisopropyladenosine (PIA) in hepatic membranes. Membranes were incubated with the indicated concentrations of sodium chloride in the presence of 10 nM glucagon and $1 \mu\text{M}$ GTP. Values shown represent the means $\pm$ S.E. of triplicate incubations.

Fig. 6. GTP-dependent inhibition of glucagon-stimulated adenylate cyclase activity by N⁶-phenylisopropyladenosine (PIA) in hepatic membranes. Incubations with various concentrations of GTP were made with 10 nM glucagon and 200 mM NaCl in the presence or absence of 0.1 μ M PIA. Values shown represent the means $\pm$ S.E. of triplicate incubations.

The inhibition by PIA of the glucagon-stimulated enzyme was evident in the presence of 200 mM of sodium chloride and clearly antagonized with 0.1 mM IBMX (Fig. 7).

Discussion

Relatively large doses of adenosine (20 μ M) have been reported to inhibit adenylate cyclase activity in hepatic membranes (10, 11, 21–24), suggesting that the inhibition was mediated via intracellular P-sites requiring an intact purine moiety, while modifications in the ribose portion had no effect on this action (1, 4). An increase in cellular cyclic AMP in the response to pharmacological concentrations (10 μ M) of adenosine and an A₂ (or Ra) agonist such as 2-chloroadenosine has been reported in isolated rat hepatocytes (13, 14). Moreover, Schültz et al. (12) have reported a stimulatory adenosine receptor (A₂ or Ra) coupled to the adenylate cyclase system in rat liver plasma membranes. In the

Fig. 7. Antagonism of N⁶-phenylisopropyladenosine (PIA) induced inhibition on glucagon-stimulated adenylate cyclase activity by 3-methyl-1-isobutylxanthine (IBMX). Hepatic membranes were incubated with or without 10 nM glucagon, 0.1 μ M PIA, 200 mM NaCl and 0.1 mM IBMX, respectively. Data shown represent the mean $\pm$ S.E. of four incubations.

present experiment, PIA, an analogue of adenosine previously shown to be a specific R-site agonist at adenosine receptors (1, 25, 26) of the adenylate cyclase system was observed to stimulate the enzyme at concentrations above the micromolar range, suggesting the presence of the stimulatory adenosine receptor (A₂ or Ra) in rat hepatic membranes.

On the other hand, the present experiment has shown the dose-dependent attenuation by PIA of the glucagon-stimulated adenylate cyclase activity. Londos and Preston (10) have reported that there was no inhibition of the glucagon-stimulated enzyme by adenosine analogues related to a specific R site agonist. Attention, however, must be paid to

their experimental conditions in which the adenylate cyclase activity has been assayed in the presence of Gpp(NH)p, a guanine nucleotide with exclusive stimulating action. In the adipocyte adenylate cyclase system, the adenosine receptor-mediated inhibition of the enzyme was observed to be absolutely dependent on GTP and amplified by sodium ions (27–29). In the present experiment, the inhibition of the glucagon-stimulated adenylate cyclase activity by PIA was apparent in the presence of a low concentration (1.0 μ M) of GTP and sodium chloride. An interaction of the monovalent cation with the guanine nucleotide-binding regulatory component, revealing hormone-induced inhibition or impairing hormone-induced stimulation has been suggested (27–29). The inhibitory effect of PIA was antagonized by IBMX, which is selective for extracellular R site receptors. These results suggest a receptor-mediated inhibitory system (25, 30, 31) for adenosine on the hepatic membrane. A higher affinity for PIA inhibiting the glucagon-stimulated activity in the nanomolar range can be compared with the far greater values (10 μ M) for adenosine (10, 11, 22–24). The IC₅₀ value for PIA (10 nM) is also compatible with those (10 to 100 nM) in the brain (32, 33), fat cells (3, 34), heart (35) and adrenocortical cells (9). The inhibition by PIA fortified with sodium chloride was observed in the presence GTP in the micromolar range, indicating the effect of the guanine nucleotide inhibitory system (Ni) linked to the enzyme.

The inhibitory effects of the glucagon-stimulated enzyme activity by PIA disappeared and reversed into a stimulatory phase with increasing concentrations of GTP (more than 10 μ M), suggesting the presence of a stimulatory (Ns) and an inhibitory (Ni) guanine nucleotide system of the enzyme in the action of PIA. The concentrations of the nucleoside required to produce a receptor-mediated inhibitory effect on the glucagon-stimulated adenylate cyclase were less than the micromolar range and of physiological significance, considering the fact that the circulating plasma levels of adenosine have been reported to be less than 2 μ M (36).

The present experiments suggest that

adenosine in the micromolar range has dual effects on the hepatic adenylate cyclase system through the stimulatory receptor (Ra or A₂) and the inhibitory receptor (Ri or A₁), depending on the cellular concentrations of GTP and/or sodium chloride.

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