ADENYLYL CYCLASE ASSAYS TO MEASURE MACROPHAGE CHEMOTACTIC PROTEIN-1 RECEPTOR FUNCTION

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ABSTRACT

Adenylyl cyclase is a membrane-bound enzyme that catalyzes the conversion of ATP to cAMP. The inhibition of adenylyl cyclase was carried out by measuring the ability of the macrophage chemotactic protein-1 to inhibit the forskolin-induced enzyme activity. Adenylyl cyclase activity in the presenc of macrophage chemotactic protein-1 was decreased compared to that in controls [2.11+/-0.15 (mean +/- SD.) vs. 6.83 +/- 0.45, activity (μmol cAMP/mg protein/min)].

Key word: Adenylyl cyclase , Macrophage chemotactic protein-1

INTRODUCTION

Many different chemokine receptors have been identified and isolated by biochemical techniques. These receptors belong to a large family of proteins, characterized by a presumed heptahelical structure, that couple with and signal via a family of heterotrimeric G proteins composed of α , β and γ subunits(1). Adenylyl cyclase is regulated by G protein complex. Adenylyl cyclase (E.C.4.6.1.1.) catalyzes the conversion of adenosine triphosphate (ATP) into cyclic AMP and pyrophosphate(2). Adenosine 3,5 monophosphate (3,5-cAMP) is an important regulatory molecule in a variety of organisms ranging from prokaryotes to at least the majority of eukaryotes (3). Analysis of the activity of adenylyl cyclase can be used for studying the function of G protein linked receptors. Adenylyl cyclase activity is regulated in either a positive or negative manner by receptor agonists, depending on whether the receptor is coupled with stimulatory (Gs) or inhibitory (Gi) G proteins (4). A number of receptors including the β-adrenergic and glucagon couple with Gs and thus stimulate adenylyl cyclase, whereas some chemokine receptors, macrophage chemotactic protein-1 (MCP-1), couple with and thus, inhibit adenylyl cyclase (5,6). Although the involvement of G-proteins in response to chemoattractant factors has been clearly established, the mechanism of action has remained elusive. The purpose of this study was to investigate the adenylyl cyclase activity assays to measure macrophage chemotactic protein-1 receptor function.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing approximately 220-240 g, aged 6-8 weeks, were used in this study. All animals survived without showing any signs of illness. The research was carried out on animals under ether inhalation anesthesia. The animals were

housed six per cage with access to water and food. The animals were maintained in an air conditioned room at 19-23°C, with a 12 h light-dark cycle, and acclimatized for three to four days before starting the experiments.

Materials

ATP, cAMP, forskolin, creatine phosphokinase and phophocreatine sucrose, EDTA, Tris-HCl leupeptin, aprotinin, phenylmethylsulfonyl fluoride, Pefabloc, HCl, NaF, GppNHP, Tris acetate, KCl, $MgCl_2$, phosphoenol pyruvate, AT P, GTP, dithiothreitol, bovine serum albumin, pyruvate kinase NaOH, $CaCl_2$, nucleotidase, aspyrase, deaminase. cAMP-2-amino-2-methyl-1-propanol NADPH were obtained from Sigma (St. Louis, MO, USA). The other reagents were purchased from Merck Chemical Company, Germany. All of the chemicals were guaranteed-grade reagents and were used without further purification. All solutions were prepared by distilled-deionised water.

Preparation of membranes

Ventricular membrane preparation were made up of ten male rats who were scarified by decapitation and immediate excision of the heart. Their hearts were placed in ice-cold buffer (0.30M sucrose, 0.1 mM EDTA, 5.0 mM Tris-HCl, pH 7.2). Portions (0.2- 0.5 g) from the ventricular apex were minced and then homogenized in ice-cold buffer (1.5, w/v). The homogenate was filtered and then centrifuged for 10 min at 1000 g. cells transfected with chemokine receptors were resuspended to a final concentration of 2×10^8 cells/ml in 40 mM Tris-HCl buffer, pH 7.2, containing 10 μg/ml each of leupeptin and aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 0.05 mM Pefabloc and 1 mM EDTA. The cells were placed in a nitrogen cavitation chamber under 300 psi of pressure at 5°C for 15 min. The lysed cells were then centrifuged for 15 min at 5°C The cell pellet, which consisted of cell debris and nuclei, was discarded and the supernatant was centrifuged at 45000g for 30 min. After centrifugation the pellet was removed and resuspended to a final concentration of 1 to 2 mg/ml in lysis buffer and stored at -70°C until enzyme activity was measured. Membranes prepared by cells stably transfected with the chemokine receptor were stimulated with forskolin in the presence and absence of increasing amounts of MCP-1 (0.0 to 10 μM), to illustrate the calculation of adenylyl cyclase activity. Assay conditions were as follows. For each assay set up the following additional samples into tubes were used. Control, forskolin (10 μM) + MCP-1 (2 μM), forskolin (10 μM) + MCP-1 (4 μM), forskolin (10 μM) + MCP-1 (6 μM) forskolin (10 μM) + MCP-1 (8 μM), and forskolin (10 μM) + MCP-1 (10 μM).

Adenylyl cyclase Activity Assay

Measurement of adenylyl cyclase activity was performed according to the procedure described by Wiegn (7). A volume of 5 μl of H_2O, 0.1 mM NaF, 0.001 mM GppNHP, 0.01 mM forskolin was added to each reaction tube and maintained at 4°C. Later 50 μl of reaction mixture (50 mM Tris acetate, pH 7.2, 30 mM KCl, 5 mM $MgCl_2$, 30 mM phosphoenol pyruvate, 4.0 mM ATP, 4.0 mM GTP, 5.0 mM dithiothreitol, 0.05% bovine serum albumin, 2.0 mg/ml pyruvate kinase) was added to each reaction tube. Finally 50 μl of incubation solution (60-90 μg protein) was added to each tube and the reaction was initiated by placing the tubes in a water bath at 37°C. After 20 min at 37°C, the reaction was stopped by the addition of 100 μl 60 mM NaOH. The reaction mixture was heated for 10 min at 100°C. A volume of 50 μl of reaction product was added to 200 μl of cleaning reaction mix (100 ml Tris-HCl, pH 7.50, 8 mM MgCl 2.3 mM $CaCl_2$, 0.03 units/ml nucleotidase, 0.3 units/ml apyrase, 0.2 mg/ml adenosine deaminase). After 20 min at 37°C, the reaction was terminated by heating at 100°C for 10 min. A volume of 500 μl of cAMP mix (60 mM imidazole-HCl, pH=7.2, 0.8 mM $MgCl_2$, 2.0 mM EGTA. 0.005% BSA, 2.0 mM inorganic phosphate, 0.1 mM glycogen, 5 μM glucose 1,6-diphosphate, 0.2 mM NADP, 0.6 mM dithiothreitol, 0.03 units/ml phosphodiesterase, 20 μg/ml glucose 6-phosphate dehydrogenase, 50 μg/ml phosphoglucomutase, 10 μg/ml, glycogen phosphorylase) was added to the reaction product. After 20 min at 37°C, 500 μl of 2-amino-2-methyl-1-propanol buffer (100 mM, pH

7.2) was added to the reaction mixtur and final concentration of NADPH was measured fluorometrically. Samples were usually assayed in duplicate. An enzyme unit was defined as the amount of enzyme that catalyzed of 1 μ mol of cAMP per min at 37°C. Specific activity was in terms of unit per mg of protein. The protein contents of various enzyme extracts relative to standard solutions of bovine serum albumin were determined by Lowry method (8).

Table 1. Comparison of adenylyl cyclase activity between incubation with MCP-1 and control

Sample	Activity (μmol cAMP/mg protein/min
Control	6.83+/-0.45
forskolin (10 μM)	10.52+/-1.33
forskolin (10 μM)+MCP-1 (2 μM)	8.72+/-0.94
forskolin (10 μM)+MCP-1 (4 μM)	7.11+/-0.85
forskolin (10 μM)+MCP-1 (6 μM)	5.38+/-0.72
forskolin (10 μM)+MCP-1 (8 μM)	3.59+/-0.65
forskolin (10 μM)+MCP-1 (10 μM)	2.11+/-0.15

The results shown are the Mean+/-S.D. of the three separate experiments.

RESULTS

Analytical calibration curves of the working standard solution were prepared by using different concentration substrates. The calibration curves were linear over the range from the detection limit of 0.0 to 12 μmol cAMP/mg protein. Figure 1 ilustrates a typical standard curve obtained with our procedure. Table 1 depicts the comparison of adenylyl cyclase activity between incubation with MCP-1 and control. The level of adenylyl cyclase activity in control was higher and statistically significant. Forskolin activation of adenylyl cyclase increased by 45% (Fig. 2). The enzyme concentrations have been kept constant throughout the measurement. Effect of the MCP-1 receptor resulted in a potent and dose-dependent inhibition of adenylyl cyclase activity (Fig. 3). MCP-1 significantly reduced basal adenylyl cyclase activity in these cells by 79.9% .

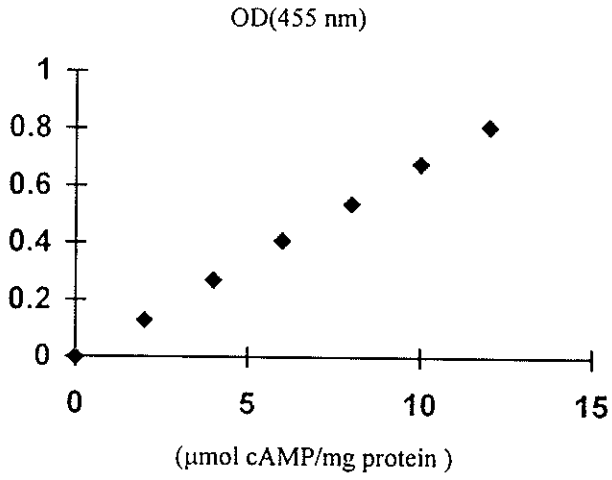

Figure 1. Analytical calibration curve of the working standard solution was prepared by using different concentration of substrates. The calibration curves were linear over the range from the detection limit of 0.0 to 12 μmol cAMP/mg protein/min. Each point represents the Mean+/- S.D. of 6 experiments.

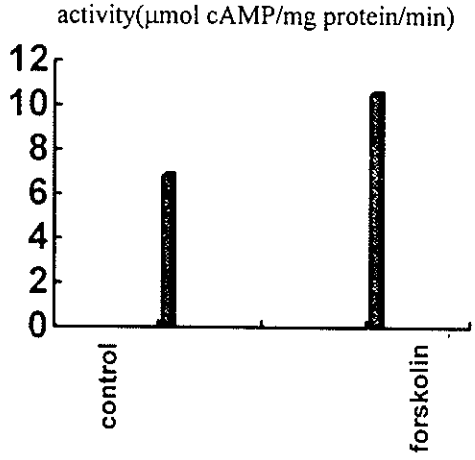

Figure 2. Forskolin activation of adenylyl cyclase increased by 45%. Each column represents the Mean +/- S.D. of 6 experiments.

DISCUSSION

The principle of the adenylyl cyclase activity assay depends on the conversion of the substrate, ATP to cAMP. Changes in the levels of the cAMP were used to measure adenylyl cyclase activity. The inhibition of adenylyl cyclase is carried out by measuring the ability of the macrophage chemotactic

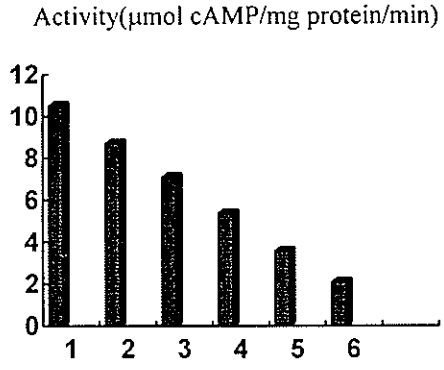

Figure 3. Activation of the MCP-1 receptor resulted in a potent and dose-dependent inhibition of adenylyl cyclase activity. MCP-1 significantly reduced basal adenylyl cyclase in these cells by 31% . 1) forskolin (10 μM), 2) forskolin (10 μM) + MCP-1 (2 μM), 3) forskolin (10 μM) + MCP-1 (4 μM), 4) forskolin (10 μM) + MCP-1 (6 μM), 5) forskolin (10 μM) + MCP-1 (8 μM) and 6) forskolin (10 μM) + MCP-1 (10 μM). Each column represents the Mean +/- S.D. of 6 experiments.

protein-1 to inhibit the forskolin- induced enzyme activity. Exposure of the adenylyl cyclase to forskolin, resulted in an elevation of adenylyl cyclase activity (Fig. 2). Exposure of adenylyl cyclase to MCP-1 resulted in a decrease of adenylyl cyclase activity (Fig. 3). Our results were in good agreement with those reported previously (9-12). Our results demonstrate that inhibition of adenylyl cyclase activity provides a sensitive and quantitative assay for MCP-1 receptor activation in cells. This study is the first demonstration of adenylyl cyclase inhibition by MCP-1 and our data lead to dose-dependent inhibition of adenylyl cyclase, which is consistent with the hypothesis that the MCP-1 receptors couple with (Gi) G-protein (2). Although inhibition of adenylyl cyclase is the most thoroughly characterized effect, the downstream effects of activation of Gi in cells are not well understood. Our data are the first biochemical studies leading to dose-dependent inhibition of adenylyl cyclase, which are consistent with the hypothesis that the MCP-1 receptors couple with Gi. Further studies are necessary to clarify this mechanism of MCP-1.

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