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# DEMONSTRATION OF ADENYLATE CYCLASE COUPLED ADENOSINE RECEPTORS IN GUINEA PIG VENTRICULAR MEMBRANES

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Adenylate cyclase in homogenates of guinea pig ventricles was inhibited by the stable adenosine analogs $N^6$-phenylisopropyl-adenosine (PIA) and 5'-N-ethylcarboxamidoadenosine (NECA). Inhibition required GTP and was enhanced by sodium ion. The maximum inhibition observed was 35.1±1.1%, the EC50 (95% confidence limits,n) for PIA and NECA were 0.20μM (0.17-0.25μM,6) and 0.66μM (0.26-1.7μM,4) respectively. 8-Phenyltheophylline (10 and 100μM) and isobutylmethylxanthine (100μM) antagonized the inhibitory effects of the adenosine analogs. These results indicate that adenosine receptors of the inhibitory type ($R_I$ or $A_1$) are present in guinea pig myocardium and may mediate some of the cardiac responses to adenosine.

Adenosine has been known for many years to be an important regulator of cardiovascular function (1, 2). The mechanisms by which adenosine exerts its negative inotropic and negative chronotropic effect in the myocardium as well as its vasodilatory action in the coronary vasculature have not been understood. It has been suggested that myocardial calcium entry channels are blocked by adenosine via a non-receptor mechanism (3). Recently adenosine receptors coupled to adenylate cyclase (AC) have been identified in the central nervous system (4,5), hepatocytes (6,7), adipocytes (6,8,9) and platelets (7,10). Both excitatory receptors ($R_A$ or $A_2$) and inhibitory receptors ($R_I$ or $A_1$) have been reported (6,11). Adenosine analogs active at these AC-coupled receptors have been shown to have negative inotropic activity in the heart (12,13). It is therefore of interest to

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determine whether AC-coupled adenosine receptors can be detected in the myocardium.

## METHODS

### 1) Materials

[$\alpha$-$^{32}$P] adenosine-5'-triphosphate (triethylammonium salt) specific activity 410 Ci/mmol and [2-$^{3}$H] adenosine-3', 5'-cyclic phosphate (ammonium salt) were obtained from the radiochemical centre, Amersham.

The following compounds were obtained from the Sigma Chemical Co., St. Louis: Adenosine-5'-triphosphate (ATP), adenosine-3', 5'-cyclic monophosphate (cAMP), adenosine deaminase, tris (hydroxymethyl)aminomethane, bovine serum albumin, creatine phosphate, creatine phosphokinase, neutral alumina. Dowex 50W 4-400 ion-exchange resin, guanosine-5'-triphosphate (GTP), isobutylmethylxanthine (IBMX).

$N^6$-phenylisopropyladenosine (PIA) was obtained from Boehringer Mannheim, 8-phenyltheophylline (8-PT) from Calbiochem. 5'-N-ethylcarboxamidoadenosine (NECA) was a gift from BYK Gulden and RO-20-1724 from Roche.

### 2) Preparation of membranes

Male guinea pigs weighing between 300-500g were killed by cervical dislocation. The heart was removed and washed with ice cold saline via the dorsal aorta. The ventricles were suspended in 0.25M sucrose containing 50mM tris HCl pH 7.5, 15mM $MgSO_4$ and homogenized using a Polytron homogenizer for two 5 second bursts separated by chilling in ice. Homogenates were filtered through two layers of gauze and centrifuged at 20,000g for 15 min. The pellets were washed in the above sucrose buffer, followed by resuspension and centrifugation. The final pellets were resuspended in the above sucrose buffer at a protein concentration of 1-3mg/ml. Protein concentration was estimated by the method of Lowry *et al* (14) using bovine serum albumin as standard.

### 3) Adenylate cyclase assay

Adenylate cyclase assays were performed in a final volume of 0.1ml at pH 7.6 and contained 50mM triethanolamine hydrochloride, 3mM MgSO4, 5mM KCl, 0.1mM EGTA [Ethylenebis (oxyethylene-nitrilo)] tetraacetic acid, 0.2mg/ml RO-20-1724, 20mM phosphorylcreatine, 10µg/ml creatine phosphokinase, 1µg/ml adenosine deaminase, 0.1mM cAMP, 1mg/ml bovine serum albumin, 0.1mM [$\alpha$-$^{32}$P]ATP. NaCl (10mM), GTP (0.1mM) and other additions were included in the assay where otherwise indicated.

Incubation was at 25°C for 12.5 min and was terminated by adding 0.2ml 1M perchloric acid to the incubation mixture. Approximately 10,000 cpm [$^{3}$H]-cAMP was added to each tube as a recovery marker. Following centrifugation to remove precipitated protein, cAMP was isolated using sequential chromatography over Dowex and neutral alumina columns (15). Recovery of cAMP was 65-75%. Under the conditions used, cAMP generation was linear both with time and with protein concentration. No [$^{32}$P]-labelled

product other than cAMP could be detected in the column eluate by thin layer chromatography. Duplicate samples agreed within ±3%.

RESULTS

1) Inhibition of adenylate cyclase by adenosine analogues

PIA and NECA produced a concentration dependent inhibition of AC (Fig. 1). In the presence of 50mM $Na^+$ and 0.1mM GTP, basal activity of the enzyme was 58.5±2.4 SEM pmol/min/mg (n=11). The maximum inhibitions produced by PIA and NECA were 35.0±1.6% (mean ±S.E.M. n=6) and 35.2±1.9% (n=4) respectively. PIA was more potent than NECA in inhibiting AC, the geometric mean $EC_{50}$ (95% confidence limits, n) for PIA was 0.20μM (0.17-0.25μM, 6) and 0.66 μM (0.26-1.7μM, 4) for NECA.

2) Effect of adenosine receptor antagonists, 8-phenyltheophylline and isobutylmethylxanthine

The effects of 8-PT and IBMX were complex, because the methylxanthines are inhibitors of cAMP phosphodiesterase (PDE) as well as antagonists at adenosine receptors (4,8). PDE activities were not completely abolished by the non-xanthine PDE inhibitor

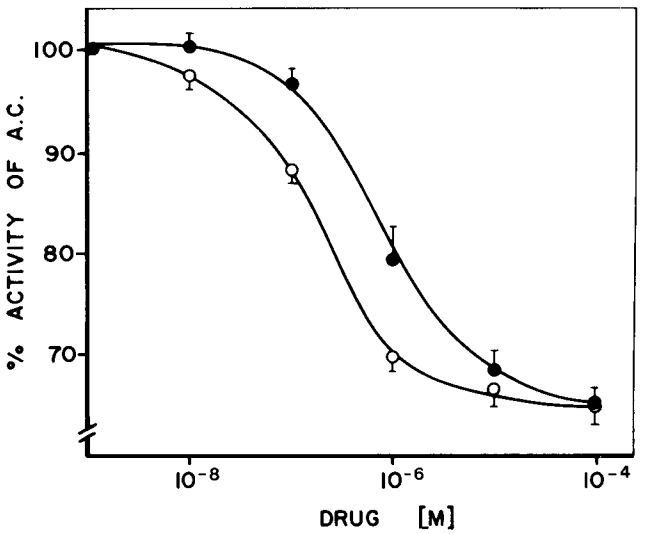


Fig. 1. Inhibition of adenylate cyclase (A.C.) by the adenosine analogs $N^6$-phenylisopropyladenosine (o-o) and 5'-N-ethylcarboxamidoadenosine (•-•) in guinea pig ventricular membranes. Basal activity of the enzyme (58.5±2.4 pmol/min/mg) was taken as 100%.

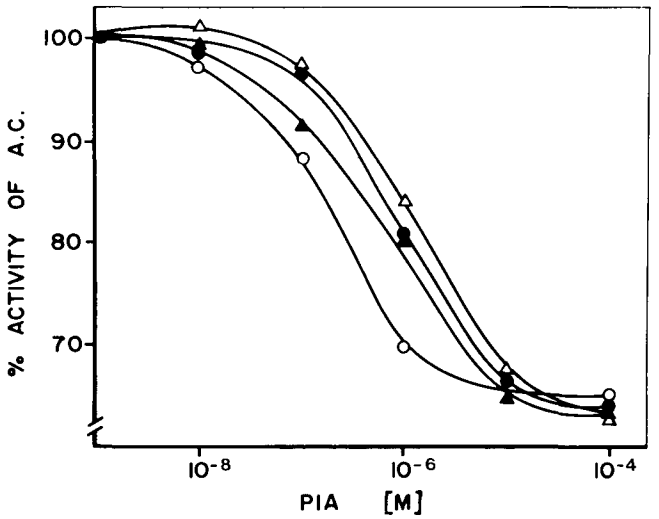


Fig. 2. Inhibition of adenylate cyclase (A.C.) by $N^6$-phenyl-isopropyladenosine (PIA)(o-o) and the antagonism of this effect by 8-Phenyltheophylline at 0.01mM (▲-▲), 0.1mM (△-△) and by isobutylmethylxanthine at 0.1mM (●-●).

RO-20-1724 at concentrations up to 0.2 mg/ml, at which stage solubility became limiting. In the presence of 8-PT (0.1 mM) or IBMX (0.1 mM),basal activity was increased to 70.0 ± 6.1 pmol/min/mg and 72.7 ±7.8 pmol/min/mg (n=3) respectively. In order to measure the effect of these antagonists on adenosine receptors, it was therefore necessary to subtract this difference in calculating results obtained in the presence of these antagonists. After subtracting the increase in basal AC activity the inhibition curve of PIA was moved to the right in a parallel manner (Fig. 2). No change in basal activity was observed in the presence of 8-PT (0.01 mM), but the dose response curve was shifted. This indicates that antagonism by the methylxanthines is not an artifact due to PDE inhibition. The maximum inhibition produced by PIA was not altered by the antagonists.

3) Effect of sodium and GTP on PIA mediated inhibition of adenylate cyclase

Basal activity of AC was increased by the addition of $Na^+$ up to 300mM. However percentage inhibition by PIA was maximal at

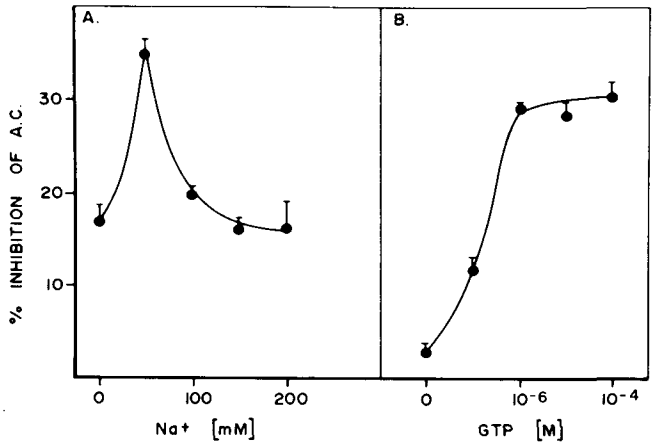


Fig. 3. a. Effect of increasing concentrations of sodium ion on the maximal inhibition of adenylate cyclase (A.C.) produced by $N^6$-phenylisopropyladenosine (PIA)
b. Effect of increasing concentrations of guanosine-5' -triphosphate (GTP) on the maximal inhibition of A.C. produced by PIA.

50mM $Na^+$ and decreased at higher concentrations (Fig.3a). Inhibition by PIA was dependent on GTP (0.1 μM-0.1 mM) with 86% of maximal effect at 1μM (Fig.3b). The geometric mean EC 50 (95% confidence limits, n) of GTP for inhibition was 0.24 μM (0.16-0.37μM, 4).

## DISCUSSION

Adenosine is a potent dilator of the coronary vasculature as well as having negative inotropic and negative chronotropic effects in the heart. Adenosine release by the myocardium is stimulated by hypoxia or exercise (16,17). The mechanism by which adenosine produces its cardiac effect is not understood. Studies in guinea pig ventricular slices and canine cardiac membranes have reported that cAMP level was increased in the presence of adenosine and a variety of adenosine analogues (18,19). However in the isolated guinea pig heart, adenosine and its analogues inhibited the increase in contractility as well as the elevated cAMP levels caused by histamine, dopamine and isoproterenol (13). Schütz and Tuisl failed to demonstrate the presence of AC

coupled adenosine receptors in guinea pig myocardium (20) and to date specific receptors for adenosine have not been identified in the heart. However these previous studies were performed before the GTP and ionic requirement for agonist inhibition of AC were recognised and this may explain the differing results.

In this report we have demonstrated inhibition of AC by stable adenosine analogs (PIA and NECA) in guinea pig myocardial membranes. PIA and NECA have both been reported to be specific 'R' site agonists (5,6,7,8) and are not active at the intracellular "P site". Moreover inhibition was antagonised by the methylxanthines 8-PT and IBMX which also are selective for extracellular "R site" receptors. Inhibition of AC in these experiments required the presence of GTP and was enhanced by $Na^+$ as has been described for other receptor-mediated inhibitory systems (8,21,22,23). Thus the observed inhibition appeared to be a receptor-mediated event rather than a direct action on the enzyme via the intracellular 'P' site (7). The receptors involved were similar to the inhibitory $R_I$ (or $A_1$) receptors described in other tissues because PIA was a more potent agonist than NECA.

The failure of previous studies to demonstrate AC inhibition by adenosine analogs in guinea pig myocardial membranes was possibly due to the use of $Na^+$ concentration not optimal for inhibition (20). These authors used 100mM $Na^+$ which has been shown to be optimal for cholinergic inhibition of AC in rabbit myocardium (24). The effect of $Na^+$ on AC inhibition by adenosine analogs in guinea pig myocardium is unusual in being maximal at 50mM and decreasing at higher concentrations. In other systems maximum inhibition was observed between 100-150mM $Na^+$ without a decrease at concentrations up to 200mM (25,26).

The findings indicate the presence of inhibitory adenosine receptors coupled to AC in guinea pig myocardium. These receptors may mediate some of the known effects of adenosine in this tissue as well as possibly explaining the inhibitory action of adenosine on catecholamine stimulated increase in inotropic effect.

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