Original Article

EFFECTS OF PHARMACOLOGICAL AGENTS ON THE ACTIVITY OF RAT KIDNEY ADENYLYL CYCLASE

Wadiah S. Backer [1] & Jalaluddin A. Khan [2]

ABSTRACT:

Objective: To study the effect of both selective and nonselective activator and inhibitory agent on adenylyl cyclase in rat kidney.

Design: Different concentrations of some pharmacological agents such as forskolin, nebularine, Ap_4A, Ap_3A and caffeine were prepared. The effects of the agents on the activity of rat kidney adenylyl were determined.

Methods: The crude extract obtained from rat kidney tissue was prepared and the specific activity of adenylyl cyclase in the crude extract was determined by using [2-H^3] ATP as substrate which produced cAMP. Different concentrations of important pharmacological agents as activators and inhibitors of enzyme were examined. The compounds selected were forskolin as a known activator of adenylyl cyclase and for comparison; nebularine, Ap_4A, Ap_3A and caffeine were used.

Results: The results obtained showed that the highest activity of adenylyl cyclase was at 100μM forskolin. The activity of enzyme was inhibited by nebularine as the concentration of agent was increased until at 50μM where the inhibition began to level off. The effect of caffeine at 10-300μM on the activity of adenylyl cyclase showed no significant effect on the enzyme activity in kidney tissue. Ap_3A showed no effect on the activity of adenylyl cyclase over the concentration range of 10-300μM, whereas, Ap_4A produced an inhibition effect on the enzyme activity at concentration 100μM.

Conclusion: The important role of cyclic nucleotides in cell-signaling and metabolism control, evoke the activities and inhibitors of cyclase to play a potential physiological effects. As forskolin is well known activator of adenylyl cyclase, other pharmacological agents such as nebularine and Ap_4A are found to be a potential inhibitors of cyclase, which highlight these compounds as treatment of mania, schizophrenia, seizure and parkinson's diseases in which an increase of cAMP concentration has been demonstrated.

KEY WORDS: Nucleotides, Adenylyl cyclase, Forskolin, Nebularine, Ap_3A, Ap_4A, Caffeine.

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INTRODUCTION

Because of the central role of cyclic nucleotides in cell-signaling and metabolic control, activators and inhibitors of the cyclases are potentially important as pharmacological agents. Some such compounds are already known and include forskolin. In this study, the activation produced by forskolin has been examined, and some other possible activators or inhibitors were examined for effect on kidney adenylyl cyclase. The effects of the compounds selected (nebularine, caffeine, Ap_3A, and Ap_4A) were compared to those of forskolin (a known activator of adenylyl cyclase). With the exception of forskolin, the compounds tested

were all purine derivatives, like cyclic AMP itself 'and ATP, the substrate of the enzyme'. Nebularine is a purine nucleoside antibiotic & has the structure purine 9-β-D-ribofuranoside. It is unique amongst the known naturally occurring purine ribosides in that it has no 6-substituent. The fungus *Lepista (Clitocybe) nebularis* produces the compound as a normal metabolite. It has high biological potency as a cytotoxin, inhibits the growth of seedlings of various species and causes mitotic aberrations in root tips. Also, it is an effective inhibitor of the multiplication of influenza **B** virus[1]. In animal cells, nebularine causes mitotic aberrations, including chromosome breaks, and the compound is highly toxic to mice and guinea-pigs[2]. The toxicity of the compound to animal cells in tissue culture is blocked by adenosine, AMP and ATP at concentrations 100-fold that of nebularine. Nebularine has also been reported to inhibit NAD^+ - dependent glucose dehydrogenase and adenosine deaminase[3].

The main source of caffeine is tea and coffee. It is a methylated xanthine and known to inhibit phosphodiesterases[4].

Ap_3A and Ap_4A have been described as alarmones, analogous to hormones in that they regulate cell metabolism, and to alarms in that they signal when all is not well and help the cell adjust to its new conditions. They are diadenosine 5',5'''-P^1P^4-tetraphosphate (Ap_4A) or its triphosphate (Ap_3A). Ames has proposed that Ap_3A represents, at least in bacteria, an alarmone that helps a cell respond to oxidative stress, imposed by agents such as hydrogen peroxide[5]. There is evidence that in animal cells Ap_4A serves as a positive regulator for DNA replication[6]. Both Ap_3A and Ap_4A are stored in large amounts in human platelets. After activation of the platelets, both of these dinucleotides are released into the extra cellular milieu where they play a role in the modulation of platelet aggregation and also in the regulation of the vasotone[7]. Similarly to Ap_3A, Ap_4A is degraded in rat plasma to ATP and AMP, and not to two ADP molecules, and these nucleotides are further degraded through adenosine[8].

MATERIAL AND METHODS

Chemical and animal sources

All biochemicals including cyclic nucleotides and column chromatography materials were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [2^3H]adenosine-5'-triphosphate (ammonium salt) was obtained from Amersham International plc (Amersham, Buckinghamshire, U.K.). The high efficiency 'Ready Safe' scintillation cocktail, containing phenylxylylethane surfactant, used for aqueous samples was obtained from Amersham also.

The source of mammalian tissue used in this study was male Wister rats. The average weights were 250-350g. They were randomly selected from a rat colony bred from animals obtained in 1976 from Olac Ltd, U.K. and maintained in the experimental animal unit of King Fahad Medical Research Center, Jeddah. They were housed in an air- conditioned room at 24°C and maintained on a 12hr dark/light cycle. Animals were fed on standard food produced by Grain, Silos and Flour Mills Organization, Western Province Saudi Arabia.

Extraction of adenylyl cyclase activity

Weighed tissues samples were placed on ice and cut into small pieces, then homogenized in ice-cold 45mM Tris-HCl (pH 7.4) containing 6mM 2-mercapto-ethanol, 8mM theophylline and 0.25M sucrose for a total of 3min, in three 1 min bursts, at maximum speed (1500 rpm). For each g of tissue, 9ml of buffer were used. The homogenate was centrifuged at 3000g for 10min at 2°C. The precipitate was discarded, and the supernatant was recentrifuged at 150,000g for 40min at 2°C. The final precipitate was resuspended in 2-3ml of 45mM Tris-HCl (pH 7.4) containing 6mM 2-mercaptoethanol and 8 mM theophylline (in experiment of caffeine 8mM theophylline was not added to the Tris-HCl buffer to avoid having two methylated xanthines present in the same experiment). It was then divided into two parts, one was assayed for adenylyl cyclase activity and the other was used for determination of protein[9].

Adenylyl cyclase assay

The procedure of Alvarez and Daniels[10] was used with minor modifications. In the assay incubations, 20µl of the Tris-HCl buffer (pH 7.4) was replaced by 20µl of the possible activators or inhibitors solution of different concentrations.

RESULTS AND DISCUSSION

Forskolin is well known as an activator of adenylyl cyclase activity and in the present study its effect was maximal at 100µM (Fig. 1). This is similar to the effect previously reported for kidney tissue[11,12]. The results, shown in Fig. 2, indicated a substantial inhibition by nebularine of adenylyl cyclase activity.

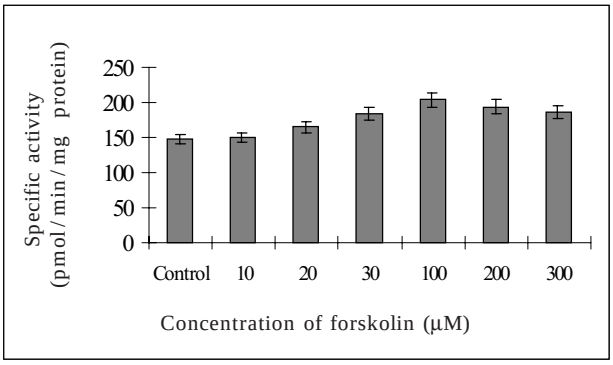

Figure-1: The stimulatory effect of forskolin on the adenylyl cyclase activity (pmol/min/mg protein) of rat kidney. 0.5mM [2-H^3] ATP was used as substrate. Incubation contained 4mM $MgSO_4$, 45mM Tris-HCl buffer at pH 7.4. Data points are each the mean of 12 replicate determination ± SD.

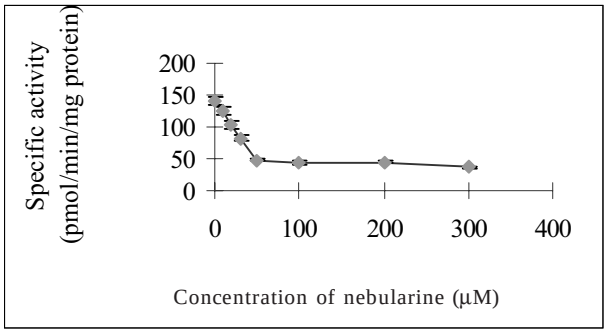

Figure-2: The inhibitory effect of nebularine on the adenylyl cyclase activity (pmol/min/mg protein) of rat kidney. 0.5mM [2-H^3] ATP was used as substrate. Incubation contained 4mM $MgSO_4$, 45mM Tris-HCl buffer at pH 7.4. Data points are each the mean of 12 replicate determination ± SD.

Initially, as the nebularine concentration was increased, there was a sharp inhibition of activity until at 50µM nebularine, the inhibition was 66%. Between 50 and 300µM nebularine, the inhibition began to level off. A kinetic study using substrate (ATP) concentrations of 0.1 to 0.5mM, and inhibitor (nebularine) concentrations of 0 ,10, 50 and 300µM showed that the inhibition was non-competitive (Fig. 3). This effect of nebularine on adenylyl cyclase activity is therefore similar to its effect on xanthine oxidase, a key enzyme of purine catabolism[13]. The inhibitory effect of nebularine on adenylyl cyclase activity could be due to an effect on the GTP- binding regulatory protein. For example, nebularine may prevent dissocia

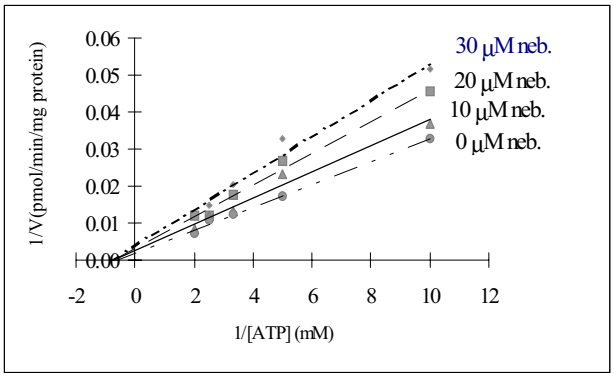

Figure-3: Lineweaver-Burk plot of adenylyl cyclase kinetics in the presence of nebularine. The enzyme was in 45mM Tris- HCl buffer (pH 7.4) containing 10mM theophylline. Data points are each of the mean of 12 replicate determination.

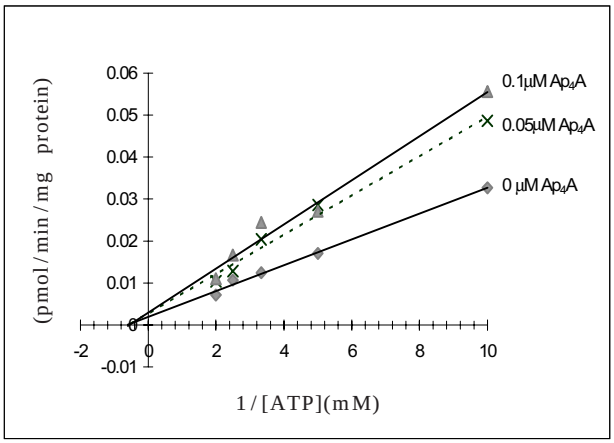

Figure-4: Lineweaver-Burk plot of adenylyl cyclase kinetics in the presence of Ap_4A. The enzyme was in 45mM Tris- HCl buffer (pH 7.4) containing 10mM theophylline. Data points are each of the mean of 12 replicate determination.

tion of the alpha subunit (α_s) of the stimulatory GTP- binding regulatory protein, G_s. Or nebularine may *in vivo* inhibit the hormone stimulating adenylyl cyclase by blocking the receptor.

The significant inhibition of adenylyl cyclase by nebularine highlights the potential importance of this compound both clinically and in the laboratory. It is possible for example, that nebularine could be of use in treating mania, schizophrenia, seizure, and Parkinson's disease, and other diseases in which an increase of cAMP concentration has been demonstrated. This compound could be considered as a selective inhibitor, since it has a high potency in inhibiting the activity of adenylyl cyclase.

The effect of caffeine was examined at 10-300μM on the activity of adenylyl cyclase (Table-I). The results showed no significant effect on the enzyme activity in kidney tissue. This result is in contrast to the reports of Sheppard[14] and Jakobs *et al*.[15] that methylxanthines in high concentrations inhibit adenylyl cyclase in some tissues. Furthermore, caffeine is known to inhibit the cAMP-induced activation of adenylyl cyclase by blocking signal transduction and not by acting directly on the enzyme[16]. Oyama and Kubota[17] found that caffeine inhibited the cation-induced activation of adenylyl cyclase in the saponin-treated cells, of the cellular slime mold *Dictyostelium discoideum*. Stimulation of striatal adenylyl cyclase by forskolin is not however significantly affected after chronic caffeine ingestion in mice[18].

This result indicates that the daily intake of caffeine in drinks such as tea, coffee, and cola

TABLE-I: The effect of caffeine on adenylyl cyclase activity (pmol / min / mg protein) from rat kidney. The incubation mixture contained 0.5mM [2-H^3] ATP and 4mM $MgSO_4$. Kidney tissue was extracted with 45 mM Tris-HCl buffer (pH 7.4) without containing 10mM theophylline. Data are the mean of 5rats; each determination was in triplicate ± SD

Caffeine (μM) final concentration	*Specific activity (pmol/min/ mg protein)*	*Change relative to control (%)*
0 (Control)	114±21	0
10	109±18	-5
20	113±19	-1
30	113±15	-1
100	112±16	-2
200	110±13	-4
300	114±17	-0

TABLE-II: The effect of Ap_3A on adenylyl cyclase activity (pmol / min / mg protein) of rat kidney. The incubation mixture contained 0.5mM [2-H^3] ATP and 4mM $MgSO_4$. Kidney tissue was extracted with 45mM Tris-HCl buffer (pH 7.4) containing 10mM theophylline. Data are the mean of 5rats; each determination was in triplicate ± SD

Ap_3A *(μM) final concentration*	*Specific activity (pmol/min/ mg protein)*	*Change relative to control (%)*
0 (Control)	112±15	0
10	112±11	0
20	113±14	-1
30	103±12	-8
100	104±13	-7
200	107±6	-5
300	107±8	-5

TABLE-III: The inhibitory effect of Ap_4A on adenylyl cyclase activity (pmol / min / mg protein) of rat kidney. The incubation mixture contained 0.5mM [2-H^3] ATP and 4mM $MgSO_4$. Kidney buffer (pH 7.4) containing 10mM theophylline. Data are the mean of 5 rats; each determination was in triplicate ± SD

Ap_4A *(μM) final concentration*	*Specific activity (pmol/min/ mg protein)*	*Change relative to control (%)*
0 (Control)	125±15	0
10	117±7	-6
20	115±13	-8
30	104±6	-17
50	98±4	-22
100	81±5	-35
200	146±17	+17
300	133±18	+6
1000	123±12	-2

does not affect the biochemical machinery of kidney metabolism but this does not necessarily mean that high intakes of caffeine is harmless.

Ap_3A and Ap_4A were added, separately, to adenylyl cyclase incubation mixtures. Ap_3A had no effect on the activity of adenylyl cyclase over the concentration range of 10-300μM (Table-II), whereas, Ap_4A at a concentration of 100μM, inhibited the activity of adenylyl cyclase by 36% of the control value (Table-III). The Lineweaver-Burk plot, of the substrate ATP concentration 0.1-0.5mM and Ap_4A at concentration 0.05 and 0.1mM shown in Fig. 4 indicates that Ap_4A is a non-competitive inhibitor of adenylyl cyclase. This results suggested that Ap_4A could be used, similarly to nebularine, for treatment of diseases in which cAMP accumulates and as an 'alarmone' through inhibiting a cyclic nucleotide system.

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