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Effects of Amiloride on A_1 Adenosine Receptor-Adenylyl Cyclase System in Rat Adipocytes[†]

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ABSTRACT

Amiloride is a potassium sparing duretic which specifically inhibits Na^+ channels. In the present study, we investigated the possible interaction of amiloride with A_1 adenosine receptors-adenylyl cyclase system in crude adipocytic plasma membrane fractions prepared from Sprague-Dawley rats. When the function of G_i protein (inhibitory guanine nucleotide binding protein) was assessed by determining the effects of GTP on isoproterenol-stimulated adenylyl cyclase activity, the inhibitory effect of high concentrations of GTP was not observed in the presence of amiloride. In contrast, the adenosine receptor-mediated inhibition of the enzyme activity, as determined empolying 2-chloroadenosine, was either unchanged or even more enhanced by amiloride depending on the concentrations of 2-chloroadenosine. Thus, it appears that GTP- and receptor-mediated inhibitory function of G_i proteins can be separated from one another. Receptor-mediated function of G_s protein did not appear to be significantly affected by amiloride, since the inhibition of isoproterenol-stimulated adenylyl cyclase activity by propranolol under the same conditions was not significantly altered by amiloride. The enhancement of 2-chloroadenosine-mediated inhibition of adenylyl cyclase by amiloride was maintained in the presence of 150 mM NaCl. In summary, these results suggest that amiloride interacts both with A_1 adenosine receptors and with G_i proteins in adipocytic membranes. Its binding to the A_1 adenosine receptors appears to facilitate the coupling of the receptors with G_i proteins thereby enhancing the inhibition of isoproterenol-stimulated adenylyl cyclase activity by A_1 adenosine agonist, and the direct interaction with G_i proteins appears to remove the GTP-dependent inhibitory effect on adenylyl cyclase activity.

Key Words: Amiloride, Adipocytes, Adenosine receptor, Adenylyl cyclase

INTRODUCTION

Amiloride, a potassium sparing diuretic, is a very potent and specific inhibitor of sodium transport in wide variety of cellular and epithelial transport systems (Benos, 1982; Kleyman and Cragoe, 1988*a* and *b*). Amiloride and its analogs have been reported to inhibit the Na^+ transport with different affinities on the epithelial Na^+ channel, the Na^+/H^+ exchanger and the Na^+/Ca^{2+} exchanger. For this reason, this drug has proven to be extremely useful for defining the physiological regulation of such transport system. Recently it has been shown that amiloride also affects, apart from ion transport systems, a number of receptors and enzymes including the α- and β-adrenergic receptors, the muscarinic receptors,

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the strial natriuretic receptor, adenylyl cyclase, and G proteins (DeLean, 1986; Friedrich and Burckhardt, 1988; Howard *et al.*, 1987; Nunnari *et al.*, 1987).

Extracellular adenosine receptors are linked to adenylyl cyclase in many systems. Originally two subclasses of adenosine receptor were defined on the basis of either inhibition or stimulation of cyclic AMP accumulation (Van Calker *et al.*, 1979; Londos *et al.*, 1980). Such receptors can also be defined operationally by the relative potencies of synthetic agonist analogs that favor one or the other subtype (Stiles, 1986; Schwabe, 1985). The inhibitory A_1 adenosine receptor subtype was characterized by Londos and his colleagues as the only extracellular adenosine receptor to exist in adipocytes (Londos *et al.*, 1980). The traditional systems that contain only the stimulatory A_2 receptor are the liver and platelet. Signal transduction mechanisms other than inhibition of adenylyl cyclase, such as modulation of calcium homeostasis and activation of potassium channels, have been also reported for the A_1 adenosine receptor (Bohm *et al.*, 1986; Paul *et al.*, 1990). For receptors which are linked to inhibition of adenylyl cyclase, Na^+/H^+ exchanger has been mentioned as additional biochemical and electrophysiological changes to occur (Limbird, 1988). Thus, it was expected that amiloride might interact with the adenosine receptors.

In the present study, we examined the effects of amiloride on A_1 adenosine receptor-adenylyl cyclase system in rat adipocytes.

MATERIALS AND METHODS

Chemicals

Adenosine 5'-[α-^{32}P]triphosphate ([α-^{32}P]ATP, specific activity: 3,000 Ci/mmol) and [8-^{3}H]cAMP (specific activity: 26 Ci/mmol) were purchased from Amersham International plc (Green End Aylesbury, Buckinghamshire, UK); Bovine serum albumin (BSA), fatty acid-free BSA, crude bacterial collagenase, adenosine deaminase from calf intestine, HEPES, EDTA, ATP, GTP, cAMP, creatine phosphate, creatine phosphokinase, alumina, Dowex 50 from Sigma Chemicals (St. Louis, MO, USA); All other chemicals were of analytical grade.

Animals

Male Sprague-Dawley rats weighing 170 to 230 g, which have been acclimatized in the animal care facilities of the university for more than a week, were used in all experiments. Rats were allowed free access to food and tap water, under a light-dark cycle with the light on from 6 a.m. to 6 p.m..

Preparation of isolated fat cells

Isolated fat cells were prepared according to the method of Rodbell (1964). Rats were killed by cervical dislocation around 10 a.m. to avoid any circadian variations and epididymal fat pads were quickly removed and pooled in Buffer A (118 mM NaCl, 4.74 mM KCl, 1.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 2.5 mM glucose, 25 mM HEPES, pH 7.4). These fat fads were then incubated with crude collagenase (1 mg/g fat tissues/3 ml Buffer A) at 37℃ for 1 hour in a Dubnoff shaking incubator (110 cycles/min). The isolated fat cells were gently filtered through a fine mesh silk screen. The cell suspension was centrifuged in a IEC clinical centrifuge at 1,000 rpm for 10 sec and the infranatant was removed. After four volumes of Buffer A were added to them, the tubes were centrifuged under the same conditions. This washing procedure was repeated a total of three times.

Preparation of plasma membrane fractions

Plasma membranes were prepared as described by McKeel and Jarett (1970). Fat cells were homogenized in 4 volumes of ice-cold Buffer B (0.25 M Sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) using a Potter-Elvejehm homogenizer. The resulting homogenates were then centrifuged at 16,000 rpm (15,000 x g) for 15 min in a Sorvall RC-5B centrifuge and the supernatant removed. The pellets were washed with Buffer C (50 mM Tris-HCl, pH 7.4 at 4℃ containing 1 mM $MgCl_2$) and centrifuged once under the same conditions as above. These pellets were selected for "crude membrane fractions", resuspended to the protein concentration of approx. 2 mg/ml in Buffer C, and stored in 20 μl-aliquots in a −70℃ deep freezer.

Determination of adenylyl cyclase activity

Adenylyl cyclase activity was determined by the method of Salomon *et al.* (1974). The total volume of the incubation medium was 50 μl which contained 0.1 mM [^{32}P]ATP, 0.1 mM cAMP, 4 mM $MgCl_2$, 1 mM GTP, 1 mg/ml BSA, 2 mM creatine phosphate, 25 unit/ml creatine phosphokinase, 0.5 unit/ml adenosine deaminase and 30 mM Tris-HCl (pH 7.5)(Londos *et al.,* 1978). Reaction was started by adding membrane fractions into the tubes and carried out at 37℃ for 15 min. [^{32}P]cAMP formed was separated from [^{32}P]ATP using alumina and Dowex 50 columns, and the radioactivity from [^{32}P]cAMP was counted in a Beckman liquid scintillation counter.

Determination of protein concentrations

Protein concentrations were determined by the method of Bradford using BSA as standard (Bradford, 1976).

Data analysis

Comparisions between groups were carried out using the Student *t*-test.

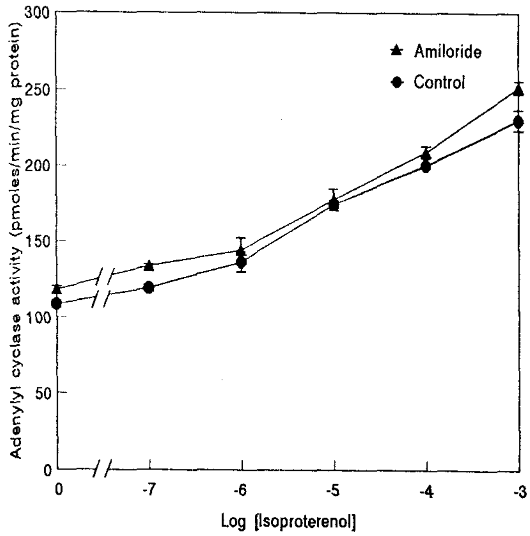

Fig. 1. Effects of amiloride on isoproterenol-stimulated adenylyl cyclase activities. Adipocytic membranes were incubated with 10^{-4} M amiloride, 10^{-7} M GTP and the indicated concentrations of isoproterenol under the "adenylyl cyclase assay" conditions for 15 min. Values are given as means±standard errors of triplicate determinations.

.RESULTS

Effects of amiloride on isoproterenol-stimulated adenylyl cyclase activity

Experiments were carried out for 20 min under the "adenylyl cyclase assay" conditions, and we found that the amounts of cAMP formed were proportional to incubation time (data not shown). Thus, in all experiments incubations were done for 15 min. As shown in Fig. 1, amiloride did not have any effects on 0 to 10^{-4} M of isoproterenol-stimulated adenylyl cyclase activity, when the enzyme activities were determined at 10^{-7} M GTP. When 10^{-4} M isoproterenol-stimulated adenylyl cyclase activities were determined at various concentraions of GTP in the presence and absence of 10^{-4} M amiloride, the enzyme activities in the absence of amilorde showed a typical biphasic curve (Fig. 2). At GTP concentrations higher than $10^{-5.5}$ M, amiloride increased the enzyme activities and the differences in the enzyme activity were statistically significant, whereas amiloride did not increase the enzyme activity at GTP concentrations less than $10^{-5.5}$ M. In all of the following reactions, 10^{-5} M GTP were included in the reaction mixture.

Effects of amiloride on actions of propranolol and 2-chloroadenosine

Activities of adenylyl cyclase can be lowered either *via* G_i employing an agonist for inhibitory receptors such as A_1 adenosine receptor or *via* G_S employing an antagonist for stimulatory receptors such as β-adrenergic receptor. To investigate the effects of amiloride on these two different pathways, isoproterenol-stimulated adenylyl cyclase activities were determined in the presence of 2-chloroadenosine, a A_1 adenosine receptor agonist or propranolol, a nonselective β-adrenergic receptor antagonist. As shown in Fig. 3, 10^{-4} M amiloride significantly enhanced the inhibition of adenylyl cyclase activities by high concentrations

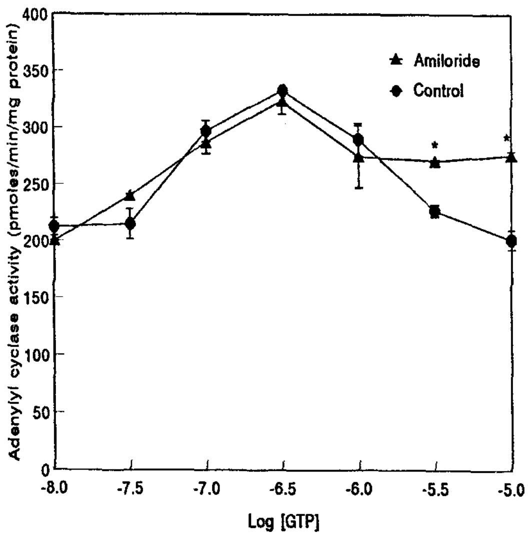

Fig. 2. Effects of amiloride on isoproterenol-stimulated adenylyl cyclase activities at various concentrations of GTP. Adipocytic membranes were incubated with 10^{-4} M isoproterenol, 10^{-4} M amiloride and the indicated concentrations of GTP under the "adenylyl cyclase assay" conditions for 15 min. Values are given as means±standard errors of triplicate determinations.

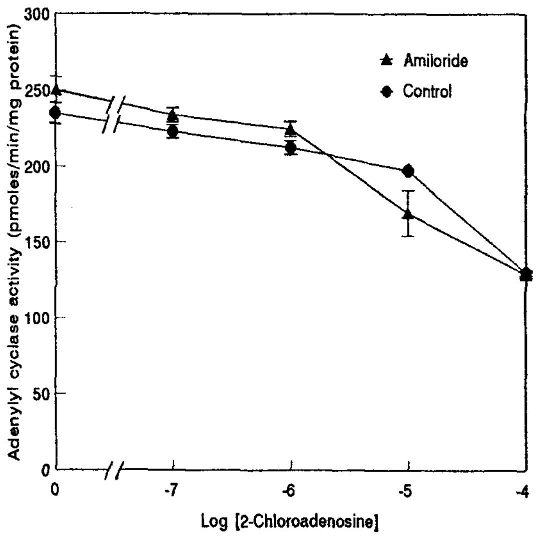

Fig. 3. Effects of amiloride on 2-chloroad~ inhibition of isoproterenol-stimulated adenylyl cyclase activities. Adipocytic membranes were incubated with 10^{-4} M isoproterenol, 10^{-4} M amiloride, 10^{-5} M GTP and the indicated concentrations of 2-chloroadenosine under the "adenylyl cyclase assay" conditions for 15 min. Values are given as means±standard errors of triplicate determinations; * stands for $p<0.05$.

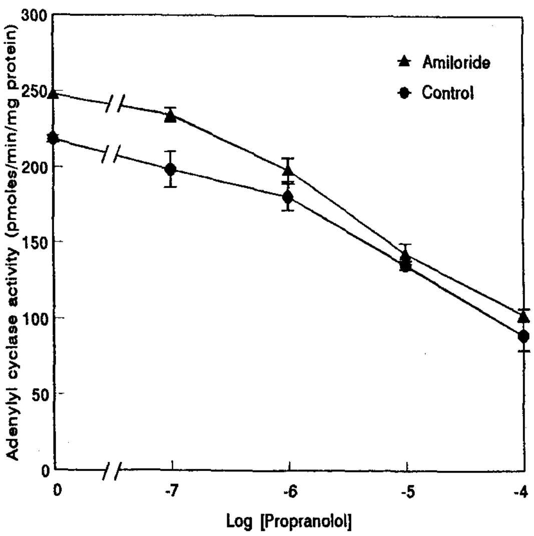

Fig. 4. Effects of amiloride on propranolol inhibition of isoproterenol-stimulated adenylyl cyclase activities. Adipocytic membrnaes were incubated with 10^{-4} M isoproterenol, 10^{-4} M amiloride, 10^{-5} M GTP and the indicated concentrations of propranolol under the "adenylyl cyclase assay" conditions for 15 min. Values are given as means ±standard errors of triplicate determinations.

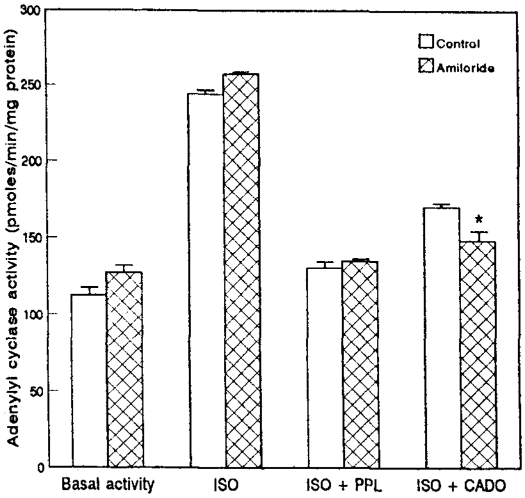

Fig. 5. Effects of amiloride on isoproterenol-stimulated adenylyl cyclase activities in the presence of cAMP regulators. Adipocytic membranes were incubated with 10^{-4} M isoproterenol, 10^{-4} M 2-chloroadenosine, 10^{-4} M propranolol, 10^{-5} M GTP and/or 10^{-4} M amiloride under the "adenylyl cyclase assay" conditions for 15 min. Values are given as means±standard errors of 5 determinations; *stands for $p<0.05$; ISO, isoproterenol; PPL, propranolol; CADO, 2-chloroadenosine.

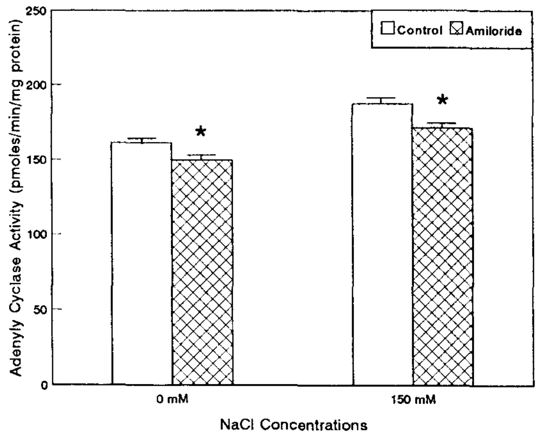

Fig. 6. Effects of amiloride on the inhibiton of isoproterenol-stimulated adenylyl cyclase activites in the presence of 150 mM NaCl. Adipocytic membranes were incubated with 10^{-4} M isoproterenol, 10^{-4} M 2-chloroadenosine, 10^{-5} M GTP and/or 10^{-4} M amiloride under the "adenylyl cyclase assay" conditions for 15 min. Values are given as means±standard errors of 6 determinations; *stands for $p<0.05$

of 2-chloroadenosine. In the absence of amiloride, 10^{-5} M 2-chloroadenosine caused a 56% reduction in 10^{-4} M isoproterenol-stimulated adenylyl cyclase activity, whereas there was a 84% reduction in the presence of amiloride (Fig. 5). Fig. 4 shows that the inhibition by propranolol was not significantly affected by the presence of 10^{-4} M amiloride at all concentrations of propranolol used.

Effects of 150 mM NaCl on amiloride actions

As shown in Fig. 6, 150 mM NaCl increased slightly the adenylyl chclase activities both in the presence and absence of amiloride. Amiloride significantly enhanced the inhibition of adenylyl cyclase by 2-chloroadenosine even at this high concentration of NaCl.

DISCUSSION

The G_S and G_i proteins exhibit very different affinities for guanine nucleotides. Thus, the G_i function can be detected by selective activation using appropriate concentrations of either GTP or p(NH)ppG (Cooper, 1982). The G_i function can be demonstrated by showing biphasic effects of GTP on isoproterenol-stimulated adenylyl cyclase activity when GTP is employed. Low concentrations of GTP promote the activation of the enzyme activity by stimulating the coupling of the β-adrenergic receptors to G_S, whereas high concentrations of GTP cause the inhibiton due to G_i. 10^{-4} M isoproterenol-stimulated adenylyl cyclase activities showed a typical biphasic curve in the absence of amiloride (Fig. 2). It was interesting to note that the GTP-mediated inhibitory effects were abolished when isoproterenol-stimulated adenylyl cyclase activities were determined in the presence of amiloride (Fig. 2).

In contrast to the loss of functional G_i, as assessed by direct activation, we found that 2-chloroadenosine, an A_i adenosine agonist, caused an enhanced inhibition of adenylyl cyclase activity in the presence of amiloride, indicating that the receptor-mediated inhibitory effect of G_i is even enhanced in the presence of amiloride contrary to what we expected. In case of β-adrenergic receptors, there were no changes in the inhibitory responses of propranolol by amiloride, indicating that receptor-mediated G_S function is not altered by amiloride. Our observation that GTP- and receptor-mediated inhibitory function of G_i can be separated has also shown in the adipocytes from streptozotocin diabetic rats (Strassheim *et al.*, 1990). As Strassheim *et al.* (1990) suggested, it is plausible that the G_i may serve two functions in the cells, one of which involves mediating a tonic inhibitory effect on adenylyl cyclase and the other the coupling to inhibitory receptors. From these results, we suggest that amiloride alters the conformation of G_i in such a way to attenuate its ability to inhibit adenylyl cyclase. However, functional coupling of G_i to inhibitory receptors is presumably enhanced and elicits a sufficiently powerful effect on the conformation of the G proteins so that it overcomes the attenuating effect.

Recently, Garritsen *et al.*, (1990*a*) reported that amiloride displaces both agonist ([^{3}H]PIA) and antagonist ([^{3}H]DPCPX) binding with a K_i value in the low micromolar range. NaCl and protons attenuated the inhibitory effect of amiloride on [^{3}H]PIA and [^{3}H]DPCPX binding without any effect on the binding to [^{3}H]PIA and [^{3}H]DPCPX, suggesting that amiloride interacts with the A_1 adenosine receptors at a site distinct from the ligand binding sites. Other study employing various protein modifiers supported this suggestion (Garritsen *et al.*, 1990*b*). Several studies on α_2-adrenergic receptors have suggested that allosteric binding sites for amiloride are on the receptor molecule itself (Wilson *et al.*, 1990; Guyer *et al.*, 1990). Anand-Srivastava (1989) have shown that amiloride interacts directly with G proteins. Amiloride did not enhance the ability of atrial natriuretic factor (ANF) to suppress adenylyl cyclase, but interact with G_o and G_i proteins. The modification of G_i proteins have shown to cause an attenuation of ANF- and angiotesin II-mediated inhibition of adenylyl cyclase activity.

In conclusion, it is suggested that amiloride might interact both with A_1 adenosine receptors and with G_i proteins in adipocytic membranes. Its binding to the A_1 adenosine receptors appears to facilitate the coupling of the receptors with G_i proteins thereby enhancing the inhibition of isoproterenol-stimulated adenylyl cyclase activity by A_1 adenosine agonist, and the direct interaction with G_i proteins appears to remove the GTP-dependent inhibitory effect on adenylyl cyclase activity.

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=국문초록=

흰쥐 지방세포에 있어서 Amiloride의 A_1 Adenosine Receptor-Adenylyl Cyclase System에 대한 작용

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박 경 선·이 명 순·김 경 환*

Amiloride는 Na^+ channels를 선택적으로 억제하는 potassium sparing diuretic이다. 본 연구에서는 amiloride와 아데노신 수용체의 상호작용을 밝히고자, 흰쥐에서 얻은 crude adipocytic membrane fractions의 adenylyl cyclase activity를 여러 조건하에서 측정하였다. 우선 GTP가 isoproterenol-stimulated adenylyl cyclase activity에 미치는 영향을 조사함으로서 G_i protein (inhibitory guanine nucleotide binding protein)의 기능을 알아보았다. 그 결과 amiloride는 높은 GTP 농도에서 isoproterenol-stimulated adenylyl cyclase의 활성을 억제하는 것을 관찰할 수 없었다. 이와는 대조적으로 amiloride 존재 하에서 2-chloroadenosine을 사용하여 아데노신 수용체를 경유한 isoproterenol-stimulated adenylyl cyclase activity가 억제되는 정도를 측정하였을 때, 2-chloroadenosine의 농도에 따라 큰 변화 없거나 오히려 억제 효과가 더욱 크게 나타났다. 그러나 위와 같은 조건하에서 propranolol에 의한 isoproterenol-stimulated adenylyl cyclase activity의 억제는 amiloride에 의해서 유의하게 변하지 않는 것으로 보아서, 수용체를 매개로 한 G_s protein의 기능은 amiloride에 의해 영향을 받지 않는 것으로 생각된다. 그리고 amiloride에 의해 증가된, 2-chloroadenosine-mediated adenylyl cyclase의 억제 효과는 150 mM NaCl 존재 하에서도 그대로 유지되었다. 이러한 결과로 보아 amiloride는 아데노신 수용체와 결합하여 G_i proteins과의 coupling을 용이하게 할 뿐만 아니라, G_i protein을 선택적으로 변화시켜 G_i protein의 GTP 의존적인 adenylyl cyclase의 억제 기능을 제거하는 두 작용을 갖는 것으로 사료된다.