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Influence of Adenosine and Magnesium on Acetylcholine Release in the Rat Hippocampus

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ABSTRACT

As it has been reported that the depolarization-induced ACh release is modulated by activation of presynaptic A_1-adenosine heteroreceptor in hippocampus and various lines of evidence indicate the adenosine effect is magnesium dependent, the present study was undertaken to delineate the role of endogenus adenosine as a modulator of hippocampal acetylcholine release in this study. Slices from the rat hippocampus were equilibrated with [^{3}H]-choline and the release of the labelled product, [^{3}H]-ACh, was evoked by electrical stimulation(3Hz, 5 V cm^{-1}, 2ms, rectangular pulses), and the influence of various agents on the evoked tritium outflow was investigated.

Adenosine, in concentrations ranging from 0.3 to 100 μM, decreased the [^{3}H]-ACh release in a dose-dependent manner without changing the basal rate of release. DPCPX(1 ~ 10 μM), a selective A_1-receptor antagonist, increased the [^{3}H]-ACh release in a dose-related fashion with slight increase of basal tritium release. And the effects of adenosine were significantly inhibited by DPCPX(2 μM) treatment. CPCA, a specific A_2-agonist, in concentration ranging from 0.3 to 30 μM decreased evoked tritium outflow with increase of basal rate of tritium release, and these effects were also abolished by DPCPX(2 μM) pretreatment. But, CGS(0.1 ~ 10 μM), a recently introduced potent A_2-agonist, did not alter the evoked tritium outflow.

When the magnesium concentration of the medium was reduced to 0 mM, there was no change in evoked ACh release by adenosine. In contrast, increasing the magnesium concentration to 4 mM, the inhibitory effects of adenosine were significantly potentiated.

These results indicate that A_1-adenosine heteroreceptor is involved in ACh-release in the rat hippocampus and the inhibitory effects of adenosine mediated by A_1-receptor is magnesium-dependent.

Key Words: Hippocampus, [^{3}H]-ACh release, Adenosine, Magnesium

INTRODUCTION

Since it was known that adenosine and related nucleotides are endogenous modulators of neuronal activity in the peripheral and central nervous system (Fredholm and Hedqvist, 1980; Burnstock and Brown, 1981; Stone, 1981; Schubert *et al.*, 1982), a large body of data on the adenosine receptors controlling the release of neurotransmitters has been accumulated (Dolphin and Archer, 1983; Jackisch *et al.*, 1983, 1984; Richardt *et al.*, 1987; Fredholm and Lindgren, 1987).

Two adenosine receptor subtypes, termed A_1 and A_2, have been differentiated based on the pharmacological profiles of adenosine agonists and antagonists at each receptor subtype (Daly *et al.*, 1983; Hamprecht and Van Calker, 1985). Inhibition by adenosine on release of various neurotransmitters including acetylcholine, norepinephrine, 5-hydroxytryptamine and glutamate in the central nervous system has been reported, and the

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receptor participitated in inhibitory effect was defined as A_1-subtype (Jackisch *et al.*, 1985; Fredholm *et al.*, 1986a; Fredholm and Lindgren, 1987).

In the hippocampus, acetylcholine (ACh) release is modulated not only by muscarinic ACh receptor (Hertting *et al.*, 1987; Choi *et al.*, 1991) but also by adenosine receptor; and the presynaptic inhibitory effect of adenosine is mediated by A_1-subytype (Jackisch *et al.*, 1984; Choi *et al.*, 1992).

On the other hand, several workers reported the existence of A_2-subtype with a heterogeneous distribution in central nervous system as well as in peripheral tissues (Bruns *et al.*, 1987; Stone *et al.*, 1988); furthermore, Fredholm *et al.*, (1986b) suggested that the A_2-receptor is present in hippocampus. Recently, Bartrup and Stone (1988) observed that A_1-receptor-mediated inhibitory effect of adenosine was eliminated in magnesium-free medium in rat hippocampus, suggesting involvement of the A_2-excitatory receptor interaction in the absence of adenosine inhibition.

However, whereas functionally distinct roles of A_1- and A_2-receptors have been established for many peripheral tissue, the physiological significance of adenosine redeptors in the central nervous system remains unclear. This study was undertaken, therefore, to characterize a role of the A_1-adenosine receptor in the evoked ACh release in the rat hippocampus, and to attempt to define, if possible, the role of A_2-receptors involved in controlling ACh release.

METHODS

Slices of 2.5~3.0 mg, 400 μm in thickness, were prepared from the hippocampus of Sprague-Dawley rats of either sex weighing 250~300 gm with a Balzers tissue chopper and were incubated in 2 ml of modified Krebs-Henseleit medium containing 0.1 μmol/L [^{3}H]-choline for 30 min at 37℃. Subsequently, the [^{3}H]-choline-pretreated slices were superfused with medium containing hemicholinium-3(10 μM) and atropine(30 nM) for 140 min at a rate of 1 mL/min. The composition (mM) of superfusion medium was 118 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 0.57 ascorbic acid, 0.03 Na_2EDTA, and 11 glucose, and the superfusate was continuously aerated with 95 % O_2+5% CO_2 with the pH adjusted to 7.4.

Collection of 5 min fractions (5 ml) of the superfusate began after 50 min of superfusion. Electrical stimulations (3Hz, 5 Vcm^{-1}, 2 ms, rectangular pulses) for 2 minutes were performed at 60 min (S_1) and 120 min (S_2). Drugs were added between S_1 and S_2 to the superfusion medium. At the end of superfusion, the slices were solubilized in 0.5 ml tissue solubilizer (0.5 N quaternary ammonium hydroxide in toluene). The radioactivity in the superfusates and solubilized tissues was determined by liquid scintillation counting (Beckman LS 5000TD). The fractional rate of tritium-outflow(5 min^{-1}) was calculated as tritium-outflow per 5 min divided by the total tritium content in the slice at the start of the respective 5-min period (Hertting *et al.*, 1980). As reported previously, the electrical stimulation of brain slices incubated with [^{3}H]-choline causes the release of [^{3}H]-acetylcholine only (Richardson and Szerb, 1974). Drug effects on the evoked tritium-outflows were evaluated by calculating the ratio of the outflows evoked by S_2 and by S_1 (S_2/S_1).

The following chemicals were used: [methyl-^{3}H]-choline chloride (72-78 Ci $mmol^{-1}$, Amersham), adenosine (Sigma), 5-(N-cyclopropyl)-carboxamidoadenosine (RBI), 8-cyclopentyl-1, 3-dipropylxanthine (RBI), atropine sulfate (Sigma) and hemicholinium-3 (Sigma). Drugs were dissolved in the medium except for forskolin and 8-cyclopentyl-1, 3-dipropylxanthine, which were initially dissolved in DMSO and then diluted in the medium.

All results are given as Mean±SEM. Significance of difference between the groups was determined by ANOVA and subsequently by Duncan test (Snedecor, 1980).

RESULTS

Effects of adenosine and 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) on [^{3}H]-acetylcholine release evoked by electrical stimulation

Hippocampal slices prelabelled with [^{3}H]-choline, a [^{3}H]-acetylcholine precursor, were superfused with the medium containing a choline up-

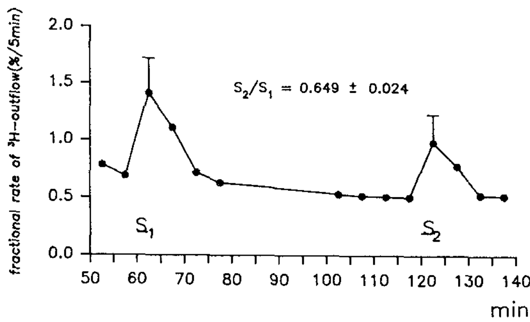

Fig. 1. Effect of 10 μM adenosine on the outflow of tritium from rat hippocampal slices preincubated with ^{3}H-choline. The slices were electrically stimulated twice for 2 min each, after 60 and 120 min of superfusion (S_1, S_2). The drug effect on the stimulation-evoked outflow of tritium are expressed by the ratio S_2/S_1. The radioactivity of the tissue at the start of experiments were 0.666±0.043 pmol. Adenosine was pretreated 15 min before S_2. The means±SEM of 4 experiments are given.

Table 1. Effect of adenosine on the electrically-evoked and basal outflows of tritium from the rat hippocampal slices preincubated with ^{3}H-choline

Drugs before S_2(μM)	n	S_2/S_1	b_2/b_1
none	7	1.055±0.026	0.633±0.023
0.3	4	1.077±0.025	0.626±0.071
1.0	4	0.952±0.037	0.670±0.033
3.0	4	0.640±0.086**	0.623±0.066
10.0	4	0.526±0.069***	0.712±0.029
30.0	7	0.402±0.034***	0.687±0.035
100.0	7	0.292±0.021***	0.683±0.028

After preincubation, the slices were superfused with medium containing hemicholinium-3 (10 μM) & atropine (30 nM) and stimulated twice (S_1, S_2). Drugs were present from 15 min before S_2 onwards at the concentrations indicated. Drug effects on basal outflow are expressed as the ratio b_2/b_1 between fractional rates of outflow immediately before S_2 (95-100 min) and before S_1 (55-60 min). Mean±SEM from number (n) of observations are given. Significant differences from the drug-free control are marked with asterisks (** = $p<0.01$ and ***=$p<0.001$).

take inhibitor, hemicholinium-3 (10 μM). And in order to eliminate the inhibition of ACh release by activating muscarinic autoreceptor, atropine (30 nM), a muscarinic antagonist was added in the superfusion medium. During superfusion, the tissue was electrically stimulated twice.

As shown in Figure 1, 10 μM adenosine decreased the electrically-evoked outflow of tritium (S_2/S_1, 0.649), but there was no change in the basal release. Adenosine in doses ranging from 0.3 to 100 μM decreased the electrically-evoked [^{3}H]-acetylcholine release in a concentration-dependent manner (Table 1).

DPCPX, a selective A_1 adenosine receptor antagonist (Bruns *et al.*, 1987), increased the evoked tritium-outflow in a dose-dependent fashion as well as the basal release (Table 2).

To ascertain the interaction between adenosine and DPCPX, the effects of adenosine were observed in the presence of the DPCPX. Both drugs were added to the superfusion medium 15 min before S_2. Fig. 2 depicts the effects of adenosine on DPCPX-treated slices as compared with those of non-treated group. The decrements of tritium-outflow were significantly inhibited by DPCPX.

Influence of magnesium ion on [^{3}H]-acetylcholine release

In order to see whether the adenosine effects are modulated by magnesium concentration, the effects of adenosine were examined in the differ-

Table 2. Effect of 8-Cyclopentyl-1, 3-dipropylxanthine (DPCPX) on the electrically-evoked and basal tritium-outflows from the rat hippocampal slices preincubated with ^{3}H-choline

Drugs at S_2(μM)	n	S_2/S_1	b_2/b_1
Control	17	0.836±0.025	0.642±0.013
DPCPX 1	4	0.840±0.029	0.704±0.032
2	7	0.870±0.025	0.710±0.019*
5	4	0.923±0.037	0.703±0.033
10	4	0.980±0.006**	0.667±0.052

Significant differences from drug-free control are marked with asterisks (*=$p<0.01$, **=$p<0.001$). Other legends are the same as in Table 1.

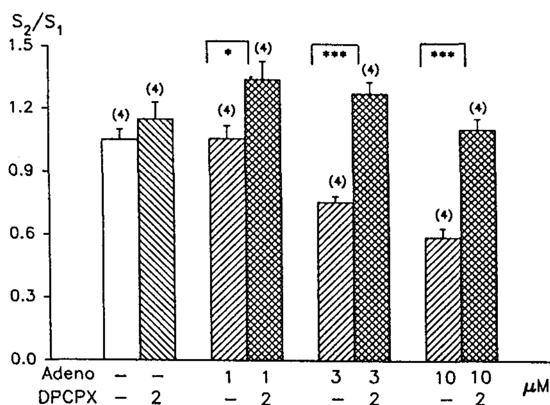

Fig. 2. Influence of 8-cyclopentyl-1, 3-dipropyl-xanthine (DPCPX) on the effect of adenosine (Adeno) on the electrically-evoked tritium outflow from the rat hippocampal slices. Asterisks indicate the significant difference (*=$p<0.05$ and ***=$p<0.001$) between groups. Other legends are the same as in Fig. 1.

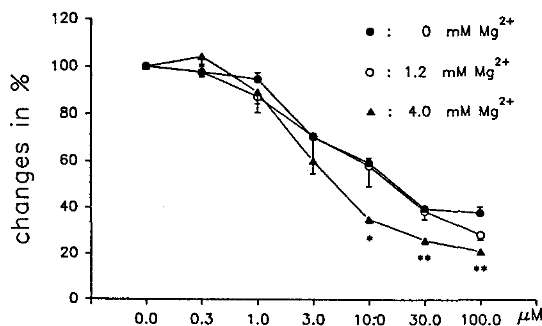

Fig. 3. The effects of adenosine on the evoked tritium outflow as related to varying Mg^{2+} concentrations. Mean changes of S_2/S_1 from the values of the control and standard error from 4 to 7 experiments are depicted. Asterisks indicate the significant difference from the 1.2 mM Mg^{2+} group (*=$p<0.05$ and **=$p<0.01$). Other legends are the same as in previous figures.

ent magnesium concentration in superfusion medium. Adding magnesium to the perfusates, in concentration of 0, 0.4 and 4.0 mM, did not show any changes of evoked tritium-outflow and basal

Table 3. Effects of varying magnesium concentration on the electrically-evoked and basal tritium-outflow from the rat hippocampal slices preincubated with ^{3}H-choline

	n	S_2/S_1	b_2/b_1
control	4	1.004±0.066	0.705±0.033
Mg^{++} 0 mM	5	1.031±0.066	0.736±0.017
0.4 mM	5	1.096±0.113	0.711±0.022
4.0 mM	4	1.095±0.095	0.668±0.050

Magnesium concentration were changed from 45 min before S_2 onwards. Other legends are the same as in Table 1

Table 4. Effect of 5-(N-cyclopropyl)-carboxamidoadenosine (CPCA) on the electrically-evoked and basal tritium-outflows from the rat hippocampal slices preincubated with ^{3}H-choline

Drugs at S_2(μM)	n	S_2/S_1	b_2/b_1
None	7	1.055±0.026	0.663±0.023
CPCA 0.3	4	0.708±0.007***	0.730±0.011**
1	4	0.701±0.030***	0.681±0.017
10	4	0.308±0.015***	0.713±0.013*
30	4	0.357±0.026***	0.802±0.022***

Significant differences from the drug-free control are marked with asterisks (*=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$). Other legends are the same as in previous tables.

release (Table 3). The adenosine effect on [^{3}H]-ACh release was not significantly affected by reducing the Mg^{2+} concentration to 0 mM. In contrast, however, increasing the magnesium concentration to 4 mM enhanced the adenosine effects inhibiting [^{3}H]-ACh release (Fig. 3).

Interactions of 5-(N-cyclopropyl)-carboxamido-adenosine (CPCA), CGS 21680C and DPCPX on [^{3}H]-acetylcholine release

Because, DPCPX, a specific A_1-antagonist, increased the basal and evoked [^{3}H]-acetylcholine release (Table 2), and the adenosine effect was potentiated by 4.0 mM magnesium, the involvement

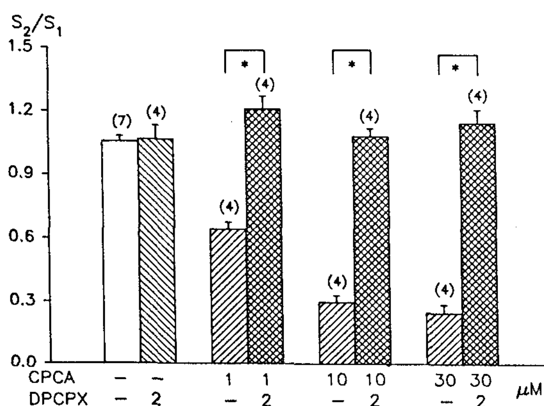

Fig. 4. Influence of DPCPX on the effect of CPCA on the electrically-evoked tritium outflow from the rat hippocampus. Asterisks indicate the significant difference (*=$p<0.001$) between groups. Other legend are the same as in previous figures.

Table 5. Effect of CGS 21680C (CGS) on the electrically-evoked and basal tritium-outflows from the rat hippocampal slices preincubated with ^{3}H-choline

Drugs at S_2(μM)	n	S_2/S_1	b_2/b_1
Control	13	1.067±0.019	0.667±0.015
CGS			
0.1	4	1.052±0.053	0.669±0.008
0.3	8	1.096±0.043	0.683±0.014
1.0	7	1.141±0.035	0.730±0.052
3.0	7	1.057±0.057	0.702±0.029
10.0	4	0.998±0.050	0.733±0.033

Legends are the same as in Table 1.

of A_2-receptor in the [^{3}H]-ACh release was suggested; and thus, the effects of CPCA and CGS 21680C were investigated. As shown in Table 4, CPCA, a specific A_2-agonist (Bruns *et al.,* 1986) in doses ranging from 0.3 to 30 μM decreased evoked [^{3}H]-ACh release in a dose-related fashion, but the basal rate of release increased. But, CGS 21680C, a recently introduced potent A_2-agonist (Hutchison *et al.,* 1989), did not alter the evoked- and basal tritium outflow (Table 5).

To ascertain whether the decreasing CPCA effects are mediated by A_1-receptor, the effects of CPCA were examined in the presence of DPCPX. As depicted in Fig. 4, the decrements of tritium-outflow by CPCA were completely abolished by 2 μM DPCPX.

DISCUSSION

It is well established that adenosine is one of the potent neuromodulators with multiple actions upon the physiology and biochemistry in the central nervous system, exerting mainly depressant action on neuronal excitement (Phillis and Wu, 1981; Dunwiddie, 1985). The effects of adenosine are mediated by specific receptors, which can be subdivided into A_1- or A_2-receptors according to their ability to either inhibit or stimulate adenylate cyclase (Van Calker *et al.,* 1979; Londos *et al.,* 1980), and both types are found to exist in the rat hippocampus (Fredholm *et al.,* 1986b). Inhibition by adenosine of release of vFarious neurotransmitters including acetylcholine, norepinephrine and glutamate in the hippocampus has been reported, and the presynaptic receptor participated in the inhibitory effect of adenosine is defined as A_1-subtype (Jackisch *et al.,* 1983, 1985; Jonzon and Fredholm, 1984; Fredholm *et al.,* 1986a).

The present study showed that adenosine inhibits the electrically-evoked release of [^{3}H]-ACh from the rat hippocampal slice. This result is in accordance with other reports that R-N^6-(2-phenylisopropyl)adenosine and N^6-cyclopentyladenosine decreased the electrically-evoked release of acetylcholine in the rat hippocampus (Duner-Engström and Fredholm, 1988; Choi *et al.,* 1992). Moreover, 8-cyclopentyl-1,3-dipropylxanthine, a selective A_1-receptor antagonist, increased the electrically-evoked tritium outflow and inhibited the effect of adenosine. These facts indicate the adenosine effect is mediated by A_1-receptor in rat hippocampus.

The existence of A_2-receptor in the brain tissue is now widely accepted (Williams, 1989; Bruns, 1990), but on the presence of A_2-receptor in the hippocampal tissue controversy still lingers. Fredholm *et al.,* (1982, 1983) described both types

of adenosine receptors mediated either increases or decreases of cAMP accumulation in hippocampal slices. In contrast, Yeung and Green (1984), working with tissue homogenates, concluded that only A_1-receptors are present in the rat hippocampus, whereas in the striatum both A_1- and A_2-receptors are functionally relevant. In experiments on rat cortical slices, Spignoli *et al.*, (1984) have shown that the A_2-specific agonist 5′-N-ethylcarboxamidoadenosine increased, whereas the A_1-specific agonist N^6-cyclohexyladenosine decreased the electrically-evoked acetylcholine release. Thus, both receptor subtypes seem to be involved in the modulation of acetylcholine release in the rat cortex. In this present study, it was attempted to see whether A_2-receptors are involved in ACh release in rat hippocampus, by observing the effects of 5-(N-cyclopropyl)-carboxamidoadenosine (CPCA) and CGS 21680C (CGS), recently introduced specific A_2-agonists, on the evoked tritium outflow. These findings that CPCA and CGS did not increase but CPCA strongly inhibited the evoked hippocampal acetylcholine release and that effects of CPCA were completely inhibited by DPCPX, a specific A_1-antagonist, may be considered to be in line with the view of Yeung and Green (1984) that in the hippocampus, at least at the level of the cholinergic nerve terminal, only A_1-adenosine receptor subtype seems to be functionally relevant.

On the other hand, since it was known that magnesium enhanced the binding of cyclohexyladenosine, an adenosine analogue, to adenosine receptors magnesium has been widely used as a tool for studying the involvement of adenosine receptors in physiological events (Goodman *et al.*, 1981; Yeung and Green, 1984; Yeung *et al.*, 1985). In electrophysiological experiments on rat hippocampal slice, Bartrup and Stone (1988) have shown that high magnesium medium enhanced, whereas magnesium-free medium greatly attenuated, the potency of adenosine in reducing orthodromically evoked population potentials elicited in area CA1, suggesting that magnesium is needed in the A_1-mediated inhibitory effcts of adenosine. However, there is no reports about the influence of magnesium upon the adenosine effects controlling neurotransmitter release. The present experiment shows that magnesium itself did not affect the basal and evoked rates of tritium release. In interaction experiments, the concentration response relation for adenosine were observed in the presence of high or low magnesium. High magnesium(4mM) significantly potentiated the inhibitory effects of adenosine, but magnesium-free medium did not affect the adenosine effect. These facts suggest that the inhibition of ACh release by adenosine was also magnesium-dependent likewise electrophysiological effects of adenosine. There are, however, evidence that the NMDA receptors are also involved in the magnesium effects (Mayer *et al.*, 1984; Nowak *et al.*, 1984; Mody *et al.*, 1987); hence, further studies are required to clarify the mechanism of the ACh release mediated by the interaction of adenosine and magnesium.

Overall, the results of the present study indicate that the decrement of the evoked ACh release by adenosine in the rat hippocampus is mediated by A_1-heteroreceptors and that the inhibitory effect of adenosine is magnesium-dependent.

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=국문초록=

흰쥐 해마에서 Acetylcholine 유리에 미치는 Adenosine 및 Magnesium의 영향

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흰쥐 해마 (hippocampus)에서 acetylcholine(ACh) 유리에 미치는 adenosine 및 이에 미치는 magnesium의 역할에 관한 지견을 얻고자하여 [^{3}H]-choline으로 평형시킨 해마 slice를 사용하여 [^{3}H]-ACh 유리에 미치는 여러가지 약물들의 영향을 관찰하였다.

Adenosine *0.3~100 μM)은 전기자극 (3 Hz, 5 Vcm^{-1}, 2 ms, rectangular pulses)에 의한 [^{3}H]-ACh 유리를 용량 의존적으로 감소시켰다. A_1-adenosine 수용체 차단제인 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX, 1-10 μM)은 용량 의존적으로 [^{3}H]-ACh 유리를 증가시켰으며, adenosine과 2 μM DPCPX 동시 투여시 adenosine의 효과가 억제됨을 볼 수 있었다.

A_2-수용체 흥분제인 5-(N-cyclopropyl)-carboxamidoadenosine (CPCA, 0.3~30 μM)은 자극에 의한 [^{3}H]-ACh 유리를 용량의존적으로 감소시켰으며, 이 역시 2 μM DPCPX 동시 투여시 그 효과가 차단됨을 관찰할 수 있었다. 그러나 또다른 A_2-흥분약인 CGS 21680C는 [^{3}H]-ACh 유리에 별다른 영향을 미치지 못하였다.

한편 관류액내의 magnesium 농도를 변화시켰을때 magnesium 그 자체로는 [^{3}H]-ACh 유리에 별다른 변화가 없었으며, magnesium을 4 mM로 증가시켰을때 adenosine의 효과가 크게 강화되어 용량 반응 곡선이 좌측으로 이동됨을 볼 수 있었다.

이상의 실험 결과로 adenosine은 흰쥐 해마의 choline 작동성 신경에 presynaptic A_1-adenosine heteroreceptor를 통하여 ACh 유리 감소를 일으키며, 이러한 adenosine 작용은 magnesium이온에 의존적임을 알 수 있었다.