Korean J. of Pharmacology
Vol. 32, No. 3, pp. 301~309, 1996

Effect of K^+-channel Blockers on the A_1-adenosine Receptor-Coupled Regulation of Electrically-Evoked Norepinephrine Release in the Rat Hippocampus

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ABSTRACT

Since it has been reported that the depolarization-induced NE release is inhibited by activation of presynaptic A_1-adenosine heteroreceptor in hippocampus, a large body of experimental data on the post-receptor mechanism of this process has been accumulated. But, the post-receptor mechanism of presynaptic A_1-adenosine receptor on the NE release has not been clearly elucidated yet. Therefore, it was attempted to clarify the participation of K^+-channel in the post-receptor mechanisms of the A_1-adenosine receptor-mediated control of NE release in this study.

Slices from rat hippocampus were equilibrated with ^{3}H-norepinephrine and the release of the labelled products was evoked by electrical stimulation (3 Hz, 5 VCm^{-1}, 2 ms, rectangular pulses), and the influence of various agents on the evoked tritium-outflow was investigated. Adenosine, in concentrations ranging from 1~30 μM, decreased the NE release in a dose-dependent manner, without affecting the basal rate of release. 4AP (1~30 μM), a specific A-type K^+-channel blocker, increased the evoked NE release in a dose-related fashion, and the basal rate of release is increased by 10 and 30 μM. TEA (1~10 mM), a nonspecific K^+-channel blocker, increased the evoked NE release in a dose-dependent manner without affecting basal release. The adenosine effects were significantly inhibites by 3 μM 4AP and 10 mM TEA treatment. 4AP (30 μM)- and TEA (10 mM)-induced increments of evoked NE release were completely abolished in Ca^{++} free, but these were recoverd in low Ca^{++} medium. And the effects of K^+-channel blockers in low Ca^{++} medium were inhibites and abolishes by Mg^{++} (4 mM) adding and TTX (0.3 μM) adding medium, respectively.

These results suggest that the decrement of the evoked NE-release by A_1-adenosine receptor is mediated by 4AP and TEA sensitive K^+-channel.

Key Words: K^+-channel blocker, A_1-adenosine receptor, Norepinephrine, Hippocampus

INTRODUCTION

It is well known that hippocampal neurons are equipped with A_1-adenosine receptor located on or close to the axon terminals of the neurones. The ultimate effect of activation of this receptor is a decrements of various neurotransmitters including acetylcholine, norepinephrine, 5-hydroxytryptamine and glutamate (Jackisch *et al.*, 1985; Fredholm *et al.*, 1986; Fredholm and Lindgren, 1987).

Norepinephrine (NE) release from rat and rabbit hippocampus can be inhibited by pre-

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synaptic α_2-adrenergic receptor (Jackisch *et al.*, 1984, 1985; Hertting *et al.*, 1987), opiate receptor (Jonzon and Fredholm, 1985), or A_1-adenosine receptor (Jonzon and Fredholm, 1984; Fredholn and Lindgren, 1988), but the underlying mechanisms of such presynaptic control of transmitter release are incompletely understood (Starke, 1987; Fredholm and Dunwiddie, 1988).

On the other hand, adenosine has been shown to modulate ion-fluxes through the membrane and second messenger system as well as transmitter release through a variety of receptor-mediated mechanisms (Williams, 1989). There are reports that the inhibitory effects by adenosine have been attributed to both inhibition of calcium conductance (Proctor and Dunwiddie, 1983; Madison *et al.*, 1986; Dolphin *et al.*, 1986) and activation of potassium channels (Dunwiddie, 1985; Trussell and Jackson, 1985). But, the involvement of ionic conductance system controlling NE release by the presynaptic A_1-adenosine receptor remains unclear until now.

Recently, potassium channels may exist in as many as twenty or more separate types in the central nervous system, and the pharmacologic and therapeutic significance of which are realized (Rudy, 1988; Robertson and Steinberg, 1990). And, there has been the progress in the influence of potassium channel modulators upon the release of neurotransmitters in the central nervous system. The release of NE from hippocampus is increased by 4-aminopyridine (4AP) (Schoffelmer and Mulder, 1983; Huang *et al.*, 1988; Hu and Fredholm, 1991; Jackisch *et al.*, 1992), tetraethylammonium (TEA) and -dendrotoxin (α-DTX) (Allgaier *et al.*, 1993), and the presynaptic effects of α_2- and A_1-receptor simulation are reduced by these drugs (Okada and Ozawa., 1980; Schoffelmer and Mulder, 1983). Moreover, Jackisch *et al* (1992) insisted that the hippocampal α_2-autoreceptor activation leads to opening of presynaptic K^+-channel, and Allgaier *et al* (1993) insisted the α_2-autoreceptors in rat hippocampus could actually be coupled to K^+-channels. However, there are reports that the hippocampal presynaptic A_1- and α_2-receptor do not primarily regulate 4AP-dependent (Fredholm, 1990; Hu and Fredholm, 1991) and TEA- or α-DTX-dependent (Allgaier *et al.*, 1993) K^+-channels.

The present study, therefore, was designed to delineate the role of K^+-channels in the evoked NE release in the rat hippocampus, and to define the involvement of K^+-channels in post-receptor mechanisms of A_1-adenosine receptor.

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-NE for 30 min at 37 ℃. Subsequently, the ^{3}H-NE-pretreated slices were superfused with medium containing desipramine (1 μM) and yohimbine (1 μM) for 150 min at a rate of 1 ml/min. The composition (mM) of superfusion medium was 118 NaCl, 4.8 KCl, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 0.57 ascorbic acid, 0.03 Na_2 EDTA, and 11 glucose, and the superfusate was continuously aerated with 95% O_2+5% CO_2, the pH adjusted to 7.4.

Collection of 5 min fractions (5 ml) of the superfusate began 50 min after the superfusion. Electrical stimulations (3 Hz, 5 Vcm^{-1}, 2 ms, rectangular pulses) for 2 minutes were performed at 60 min (S_1) and 125 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 counter (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). 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). And the influences of drugs on the basal outflow are expressed at the ratio b_2/b_1 between fractional rates of outflow immediately before S_2 (120~125 min) and S_1 (55~60 min). The following chemicals were used: l-[7,8-^{3}H]-nora-

drenaline (30~50 Ci $mmol^{-1}$, Amersham), adenosine (RBI), tetraethylammonium bromide (Sigma), 4-aminopyridine (Nakarai), desipramine HCl (Sigma), yohimbine HCl (Sigma), tetrodotoxin (RBI). Drugs were dissolved in the medium except tetrodotoxin which was initially dissolved in DMSO and then diluted with 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 and Cochran, 1980).

RESULTS

Effects of adenosine on ^{3}H-norepinephrine release evoked by electrical stimulation

Hippocampal slices prelabelled with ^{3}H-NE were superfused with the medium containing desipramine (1 μM), a NE uptake inhibitor. And in order to eliminate the inhibition of NE release by activating adrenergic autoreceptors, yohimbine (1 μM), a α_2-adrenergic antagonist, was added in the superfusion medium. During superfusion, the tissue was electrically stimulated twice.

As shown in Fig. 1, 10 μM adenosine decreased the electrically-evoked outflow of tritium (S_2/S_1, 0.710±0.014) with slight decrease in the basal release. Adenosine, in doses ranging from 1 to 30 μM decreased the electrically-evoked ^{3}H-NE release in a concentration-dependent manner (adenosine effects) (Table 1).

Effects of K^+-channel blockers on ^{3}H-NE release

As shown in Table 2, 4AP, a specific A-type K^+-channel blocker, in doses ranging from 1 to 30 μM increased the evoked NE release in a dose-related fashion. Moreover, basal outflow of NE is significantly increased by 10 and 30 μM 4AP. TEA, a nonspecific K^+-channel blocker, in doses ranging from 1 to 10 mM, increased the e-

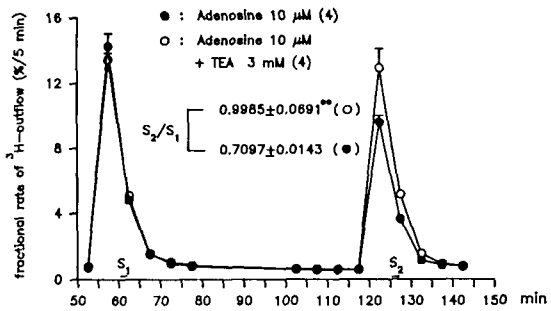

Fig. 1. A typical presentation of the tritium-outflow from the rat hippocampal slice preincubated with ^{3}H-norepinephrine. The slices were electrically stimulated twice for 2 min each, after 60 and 125 min of superfusion (S_1, S_2). The drug effect on the stimulation-evoked tritium outflow is expressed by the ratio S2/S1. The radioactivities of the tissues at the start of experiment were 1.586±0.065 (●) and 1.682±0.133 (○) pmol. Adenosine and tetraethylammonium (TEA) were added 15 min before S_2. In the parentheses are the number of the experiments. Asterisks indicate significant difference (**;$p<0.01$) between the adenosine and adenosine plus TEA-treated groups.

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

Drugs before S_2(μM)		n	S_2/S_1	b_2/b_1
none		12	0.8692±0.0391	0.8130±0.0070
Ade	1	12	0.7933±0.0145	0.7756±0.0233
	3	12	0.7591±0.0170*	0.8035±0.0293
	10	11	0.6366±0.0217***	0.7507±0.0209*
	30	12	0.6326±0.0178***	0.7522±0.0243*

After preincubation, the slices were superfused with medium containing 1 μM desipramine & 1 μM yohimbine, and then stimulated twice (S_1, S_2). Drugs were presented 15 min before S_2 at the indicated concentrations. Drug effects on basal outflow are expressed as the ratio b_2/b_1 between fractional rates of outflow immediately before S_2 (120-125 min) and before S_1 (55-60 min). Mean±SEM from number (n) of observations are given. Significant differences from the drug-free control (none) are marked with asterisks (*: $p<0.05$ and ***: $p<0.001$). Other legends are the same as in Fig. 1.

Table 2. Effect of 4-aminopyridine (4AP) on the electrically-evoked and basal outflows of tritium from the rat hippocampus

Drugs before S_2(μM)		n	S_2/S_1	b_2/b_1
none		4	0.878±0.042	0.717±0.035
4AP	1	4	0.917±0.076	0.743±0.081
	3	7	1.041±0.039	0.685±0.045
	10	4	1.329±0.025***	0.820±0.023*
	30	4	1.678±0.046***	1.178±0.087**

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

Table 3. Effect of tetraethylammonium (TEA) on the electrically-evoked and basal outflows of tritium from the rat hippocampus

Drugs before S_2(mM)		n	S_2/S_1	b_2/b_1
none		8	1.074±0.033	0.860±0.031
TEA	1	7	1.122±0.027	0.842±0.012
	3	5	1.175±0.027*	0.931±0.053
	10	5	1.343±0.025***	0.839±0.040

Legends are the same as in Table 1.

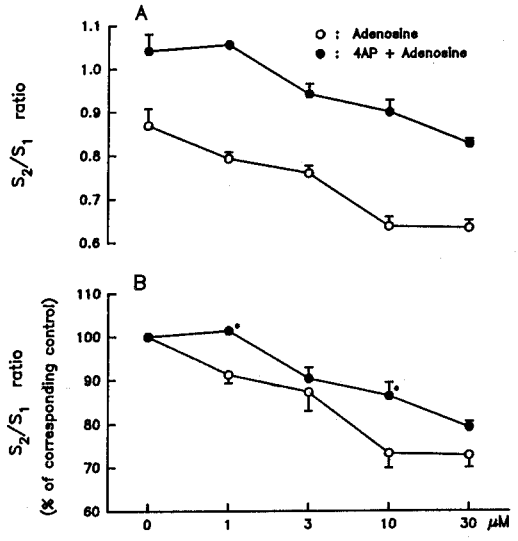

Fig. 2. Influence of the 4-aminopyridine (4AP) 3 μM upon the effect of adenosine on the electrically evoked ^{3}H-NE release from the rat hippocampal slices. A: Results are expressed S_2/S_1 ratios. Each point denotes mean±SEM from 4-8 experiments per group, but the SEM smaller than the width of the points are not shown. B: The same data but expressed are percentages of the corresponding controls. Statistically significant differences are indicated as *:$p<0.05$.

voked NE release in a dose-dependent manner, but did not change the basal release (Table 3).

Interactions of K^+-channel blockers and adenosine on ^{3}H-NE release

To ascertain whether the adenosine effects are mediated by K^+-channel modulation, the interaction of adenosine and K^+-channel blockers was then studied. As shown in Fig. 2, when the slices were treated with combination of adenosine and 4AP, 4AP completely inhibited the adenosine effects. And the dose-response curve of adenosine was shift to the right by 4AP treatment, but there was no significant difference between the slopes of regression lines (adenosine group; $y = -0.750x + 91.541$, 4AP treated group; $y = -0.659x + 97.299$).

Next, the influence of TEA on the adenosine effects was investigated. As depicted in Fig. 3, adenosine effects are not affected by 1 mM but are significantly inhibited by 3 mM TEA. To ascertain the interaction between TEA and adenosine, the slopes of three regression lines, adenosine ($y=-0.750x+91.541$), adenosine under 1 mM TEA ($y = -0.876x + 87.947$) and adenosine under 3 mM TEA ($y=-0.601x+96.725$), were compared, but there were no significant differences among the slopes.

To clarify the mechanism of the K^+-channel blockers on the evoked NE release, the effects of K^+-channel blockers were investigated in another sets of experiment in which the concentration of Ca^{++} was lowered 1.3 to 0.325 mmol/l and Mg^{++} increased from 1.2 to 4 mmol/l, respectively.

In the preliminary experiments, the electrically-evoked ^{3}H-NE release was significantly decreased by lowered external Ca^{++} from 1.3 to 0.325 mM (S_2/S_1; 0.151±0.011, n=5) compared

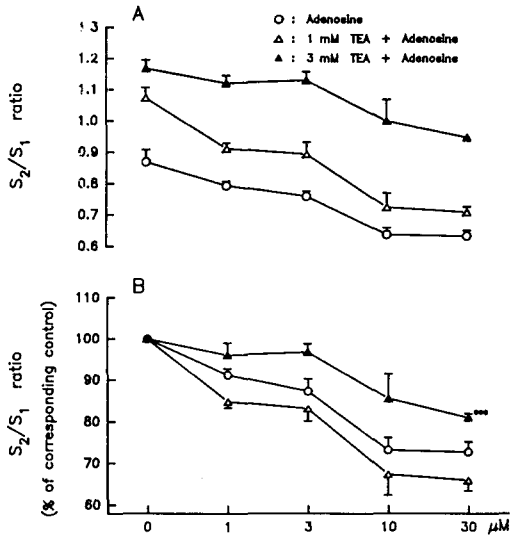

Fig. 3. Influence of the tetraethylammonium (TEA) upon the effect of adenosine on the electrically evoked ^{3}H-NE release from the rat hippocampal slices. A: Results are expressed as S2/S1 ratios. Each points denotes mean± SEM from 4-8 experiments per group, but the SEM smaller than the width of the point is not shown. B: The same date but expressed are percentages of the corresponding controls. Statistically significant differences is indicated as ***:$p<0.001$.

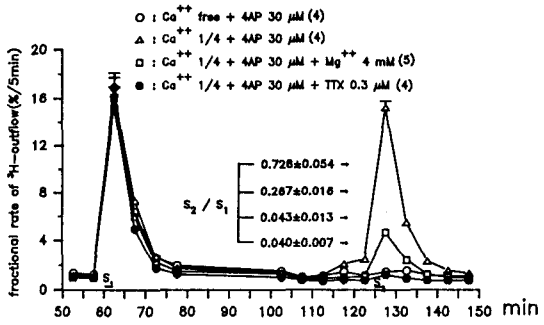

Fig. 4. The influence of Mg^{++} and tetrodotoxin (TTX) upon the 4AP-induced tritium outflow in lowered or free external Ca^{++} concentration from rat hippocampal slices prelabelled with ^{3}H-NE. The concentration of external Ca^{++} was lowered from 1.3 to 0.325 (1/4) or 0 (Ca^{++}-free) mmol/l from 15 min and Mg^{++} was increased 1.2 to 4 mmol/l from 45 min before S_2 onwards, respectively. In some experiments either 4AP or TTX was present from 15 min before S_2 onwards. The S_2/S_1 ratios were: 0.043±0.013 in Ca^{++}- free; 0.728± 0.054 in low Ca^{++}; 0.277±0.016 in low ca^{++}/ high Mg^{++}; 0.040±0.007 in low Ca^{++}/TTX. In the parentheses are the number of experiments.

with the control (S_2/S_1; 0.869±0.039, n=12). Adding the magnesium (4 mM) to the lowered Ca^{++} medium 45 min before S_2 onwards, the evoked ^{3}H-NE was more inhibited (S_2/S_1; 0.036± 0.007). As shown in Fig. 4, 4AP-induced increments of the evoked NE release was completly abolished in Ca^{++} free medium, and this inhibition was recoverd in low Ca^{++} medium with increased basal rate of release. And, increasing effects of evoked and basal rate of release by 4AP in low Ca^{++} medium were significantly inhibited and completely abolished by Mg^{++} added and TTX added medium, respectively. Next, the effects of TEA blocker on the evoked NE release were investigated in the Ca^{++} and Mg^{++} modification medium. TEA-induced evoked NE release was completely abolished in Ca^{++}- free medium but this inhibition was significantly recoverd in low Ca^{++} medium. And, this inhibitory also attenuates or abolished by adding Mg^{++} and adding TTX in medium, respectively as like as in 4AP experiments (Fig. 5).

DISCUSSION

A major aim of the present study was to investigate the effects of K^+-channel blockers to gain more information about the mechanism underlying the presynaptic inhibitory effect of adenosine. In the present study, the electrically evoked release of ^{3}H-NE from the rat hippocampal slice was inhibited by adenosine in the rat hippocampus. This result is in accordance with other reports that the electrically-evoked

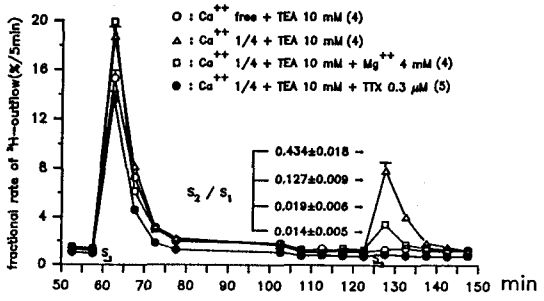

Fig. 5. The influence of Mg^{++} and TTX upon the TEA-induced tritium outflow in lowered or free external Ca^{++} concentration from rat hippocampal slices prelabelled with ^{3}H-NE. The concentration of external Ca^{++} was lowered from 1.3 to 0.325 (1/4) or 0 (Ca^{++}-free) mmol/l from 15 min and Mg^{++} was increased 1.2 to 4 mmol/l from 45 min before S_2 onwards, respectively. In some experiments either TEA or TTX was present from 15 min before S_2 onwards. The S2/S1 ratios were: 0.019±0.006 in Ca^{++} free; 0.434±0.018 in low Ca^{++}; 0.127±0.009 in low ca^{++}/high Mg^{++}; 0.014±0.005 in low Ca^{++}/TTX. In the parentheses are the number of experiments.

release of NE in rabbit (Jackisch *et al.*, 1985; Hertting *et al.*, 1987) and rat (Hu and Fredholm, 1991; Kim and Yang, 1995) hippocampus.

On the other hand, there are reports that various types of voltage-dependent K^+-channel exist in central nervous system (Rudy, 1988; Storm, 1990) and are impressive evidences that K^+-channels are involved in the neurotransmitters releasing process in striatum (Drukarch *et al.*, 1989) and hippocampus (Jackisch *et al.*, 1992; Allgaier *et al.*, 1993). Therefore, in order to confirm whether the voltage-dependent K^+-channel blockers on the evoked NE release were investigated in this study.

4AP and TEA substantially increased ^{3}H-NE release evoked with electrical stimulation. The increasing effect was abolished by Ca^{++}-free medium, but was significantly recovered in low Ca^{++} medium. And the inhibitory effect was attenuated and abolished by adding Mg^{++} and adding TTX in medium, respectively, indicating that it reflects nerve impulse-triggered and calcium-dependent release. These facts, in conjunction with the evidence that K^+-channel blockers inhibit the outward K^+-currents, indicate that the increment of transmitter release is probably due to an increase of Ca^{++} influx into the axon terminal secondarily to a prolongation of the duration of action potential. There are, however, several points not to be overlooked in the action of 4AP. In contrast to the effects of TEA, large dose of 4AP induces in itself a significant spontaneous release of ^{3}H-NE in normal and even though in low Ca^{++}medium. And also, 4AP significantly increased the evoked ^{3}H-NE (S_2/S_1: 0.267±0.016, n=5, $p<0.001$) compared to drug free control in low Ca^{++}/high Mg^{++} medium (S_2/S_1: 0.036±0.007, n=5). According to the reports that the action site of 4AP is intracellular (Rudy, 1988) and magnesium ions block the ionic channel in rat hippocampal slices (Mody *et al.*, 1987), the possibility of spontaneous and evoked release of ^{3}H-NE by 4AP are results from intracellular Ca^{++} release from the storage sites could be ruled out. But, increments of evoked and spontaneous release by 4AP were completely abolished by TTX-treated and Ca^{++}-free medium, indicating these effects are TTX-sensitive and extracellular Ca^{++}-dependent. On the basis of these findings, in conjunction with other reports that TEA blocks a wide variety of K^+-channels whereas 4AP is supposed to show some seletivity for the A current and delayed rectifier (for review see: Cook, 1988; Rudy, 1988; Castle *et al.*, 1989), indicate that the TEA- and 4AP-induced ^{3}H-NE release is mediated through different K^+-channels, respectively.

Recently, it has been clearly found that the presynaptic effects of α_2- and A_1-adenosine receptor on neurotransmitter release are coupled to K^+-channel (Schoffelmeer and Mulder, 1983; Jackisch *et al.*, 1992; Allgaier *et al.*, 1993). Therefore, in order to confirm whether the voltage dependent K^+-channels are involved in A_1-receptor mediated decrease of NE release, the influence of K^+-channel blockers upon the adenosine effects was investigated in this study.

In interaction experiments, the concentration-response relations for adenosine were observed in the presence of 4AP and TEA. 4AP and TEA significantly attenuates the inhibitory ef-

fects of adenosine. This result is in accordance with other reports that depressant effects of adenosine is blocked by 4AP in neocortex (Perkins and Stone, 1980) and hippocampus (Okada and Ozawa, 1980; Schubert *et al.*, 1986). These facts, in conjunction with the report that adenosine enhances an aminopyridine-sensitive K^+-conductance in nerve terminals and changes in Ca^{++} influx are consequantial to this (Scholfield and steel, 1988), suggest that the inhibitory effects of adenosine are coupled to the 4AP- and TEA-sensitive K^+-channels. However, Hu and Fredholm (1989, 1991) insist that the pre-synaptic A_1-receptors on rat hippocampal noradrenergic neurons do not primarily regulate 4AP dependent potassium channels, but they might act directly on a Ca^{++} conductance. Discrepancies between the present results and the above reports, may not be easily reconciled, but the possibilities accounting for the difference are that they did not observe the dose-response relationships of adenosine and of adenosine under K^+-channel blockers. And also there is evidence that the K^+-channels are coupled to α_2-adrenoceptor modulating NE release in rat hippocampus (Allgaier *et al.*, 1993). Hence, further studies are required to determine the exact mechanism in the NE release mediated by presynaptic A_1-adenosine receptor in the rat hippocampus.

Overall, the present study has shown that the decrements of the evoked NE release by A_1-adenosine receptor is coupled to 4AP- and TEA-sensitive K^+- channels and the adenosine-induced decrements are consequential to changes in Ca^{++}-influx.

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=국문초록=

흰쥐 해마에서 Norepinephrine 유리를 조절하는 A_1-adenosine 수용체의 역할에 미치는 K^+ 통로 차단제의 영향

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흰쥐 해마(hippocampus)에서 norepinephrine(NE) 유리에 미치는 A_1-adenosine 수용체의 post-receptor 기전에 K^+-통로가 관여하는지에 대한 지견을 얻고자 하여 ^{3}H-NE로 평형시킨 해마 절편을 사용하여 adenosine의 ^{3}H-NE 유리에 미치는 K^+-통로 차단제의 영향을 관찰하였다.

Adenosine(1～30 μM)은 전기자극(3 Hz, 2 ms, 5 VCm^{-1}, 구형파)에 의한 NE 유리를 용량 의존적으로 감소시켰다. K^+-통로 차단제의 하나인 4-aminopyridine(4AP, 1～30 μM)은 자극에 의한 NE 유리를 용량 의존적으로 증가시켰으며 특히 10 및 30 μM의 투여에 의해 기저 유리 또한 증가시켰다. 또 다른 K^+-통로 차단제인 tetraethylammonium(TEA, 1～10 mM) 역시 자극에 의한 NE 유리를 용량 의존적으로 증가시켰으나 이때 기저 유리에는 변화를 보이지 않았다. K^+-통로 차단제의 adenosine 효과에 미치는 실험에서는 adenosine에 의한 NE 유리 감소효과가 4AP 3 μM 동시 투여에 의해 억제되었으며, 또한 1 mM TEA에 의하여는 영향을 받지않았으나 3 mM TEA 동시 투여에 의하여는 억제됨을 볼 수 있었다. 한편 30 μM 4AP 에 의한 NE 유리 증가효과는 Ca^{++} 제거 영양액에서는 완전히 소실되었고 영양액 내의 Ca^{++}을 정상 농도의 1/4로 하였을때에는 NE 유리 억제가 어느정도 회복됨을 볼 수 있었으며 이때 기저 유리 또한 증가됨을 볼 수 있었다. 1/4 Ca^{++} 농도시에 4AP의 NE유리에 미치는 효과는 영양액내의 Mg^{++}을 4 mM로 올렸을때 크게 억제되었으며 0.3 μM tetrodotoxin(TTX) 동시 투여에 의해 완전히 차단됨을 볼 수 있었다. TEA 10 mM에 의한 NE 유리 증가효과 역시 Ca^{++} 제거 영양액에서 완전히 소실되었고 이 또한 1/4 Ca^{++} 영양액에 의하여는 회복됨을 볼 수 있었으며 Mg^{++} 증가 영양액에서는 억제, TTX 동시 투여시에는 완전히 소실되었다.

이상의 실험결과로 흰쥐 해마에서 A_1-adenosine 수용체를 통한 adenosine의 NE 유리 감소는 TEA 및 4AP에 예민한 K^+-통로가 관여하고 여기에는 세포외액의 Ca^{++}의 농도가 중요한 인자의 하나로 관여 하는 것으로 사료된다.