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Interaction of Forskolin with the Effect of N^6-Cyclopentyladenosine on [^{3}H]-Acetylcholine Release in Rat Hippocampus

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ABSTRACT

As it has been reported that the depolarization-induced acetylcholine (ACh) release is modulated by activation of presynaptic A_1-adenosine heteroreceptor in hippocampus and various lines of evidence indicate the involvement of adenylate cyclase system in A_1-adenosine post-receptor mechanism in hippocampus, it was attempted to delineate the role of adenylate cyclase system in the A_1-receptor-mediated control of ACh release in this study. Slices from rat hippocampus were incubated with [^{3}H]-choline 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.

N^6-cyclopentyladenosine (CPA), a specific A_1-adenosine receptor agonist, in concentrations ranging from 0.1 to 10 μM, decreased the [^{3}H]-ACh release in a dose-dependent manner without the changes of basal rate of release. 8-cyclopentyl-1, 3-dipropylxanthine (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 CPA effects were significantly inhibited by DPCPX (2 μM) pretreatment and the dose-response curve produced by CPA was shifted to the right.

The responses to N-ethylmaleimide (NEM, 10 & 30 μM), a SH-alkylating agent of G-protein, were characterized by increments of the evoked ACh-release and the basal release, and the CPA effect were completely abolished by NEM pretreatment.

Forskolin, a specific adenylate cyclase activator, in concentrations ranging from 0.3 to 10 μM, increased the evoked ACh-release in a dose-dependent manner and the CPA effects were inhibited by forskolin.

These results indicate that the A_1-adenosine heteroreceptor plays an important role in ACh-release via nucleotide-binding protein Gi in the rat hippocampus and that the adenylate cyclase system might be participated in this process.

Key Words: Hippocampus, [^{3}H]-ACh release, N^6-Cyclopentyladenosine, 8-Cyclopentyl-1, 3-dipropylxanthine, Forskolin, A_1-adenosine receptor

INTRODUCTION

Since it was known that neurotransmitter release is modulated by presynaptic auto-and hetero-receptor mechanisms in the central nervous system, a large body of experimental data on the post-receptor mechanism of this process has been accumulated (Markstein *et al.*, 1984; Pedata *et al.*, 1986; Limberger *et al.*, 1986; Allgaier *et al.*, 1987; Hertting *et al.*, 1987). Recently, Starke (1987) re-

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ported that forskolin and fluoride, both activators of adenylate cyclase, increase the electrically-evoked release of [^{3}H]-norepinephrine, suggesting that the activation of adenylate cyclase with subsequent increase of intracellular cAMP play the crucial role in the neurotransmitter release. And several lines of investigation demonstrated that the adenylate cyclase system plays a key role also in acetylcholine (ACh) release from neural tissues (Alberts and Ögren, 1988; Brigges *et al.*, 1988; Duner-Engström and Fredholm, 1988; Choi *et al.*, 1991).

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). Adenosine acts via two subtypes of receptors, A_1 and A_2 in central nervous system as well as in peripheral tissues (Van Calker *et al.*, 1979; Londos *et al.*, 1980), and one of important roles in the center is to act as an inhibitor of transmitter release (Fredholm and Hedqvist, 1980). In the hippocampus, ACh release is modulated not only by muscarinic ACh receptors (Hertting *et al.*, 1987; Choi *et al.*, 1991) but also by adenosine receptors (Jackisch *et al.*, 1983), and the pre-synaptic inhibitory effect of adenosine is mediated by A_1-subtype (Jackisch *et al.*, 1983, 1984). Although activation of A_1-adenosine receptor has been shown to reduce adenylate cyclase activity with decreased cAMP accumulation (Fredholm *et al.*, 1986), the involvement of adenylate cyclase system in controlling ACh release by the presynaptic A_1-adenosine receptor has not been clearly elucidated yet.

The present study, therefore, was designed to characterize the A_1-adenosine receptor in the evoked ACh release and to clarify the involvement of adenylate cyclase system in this process in the rat hippocampus.

METHODS

Slices of 2.5~3.0 mg, 400 μm in thickness, were prepared from the hippocampus of Sprague-Dawley rats weighing 250~300 gm (either sex) 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 (3 Hz, 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), and the radioactivity was counted. The determination of tritium in the superfusates and solubilized tissues was made by liquid scintillation counting (Beckman LS 5000TD). The fractional rate of tritium-outflow ($5min^{-1}$) 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 only [^{3}H]-acetylcholine (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), forskolin (RBI), N-cyclopentyladenosine (RBI), N-ethyl-maleimide (Sigma), 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 throughout this paper. Significance of difference between the groups was determined by ANOVA and subsequently by Duncan test (Snedecor, 1980).

RESULTS

Effects of N^6-cyclopentyladenosine (CPA) 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 uptake inhibitor, hemicholinium-3 (10 μM). And in order to eliminate the inhibition of ACh release by activation of muscarinic autoreceptor, a muscarinic antagonist, atropine (30 nM) was added in superfusion medium. During superfusion, the tissue was electrically stimulated twice. As shown in Fig. 1, 1 μM CPA, a specific A_1 adenosine receptor agonist (Williams *et al.*, 1986), decreased the electrically evoked outflow of tritium (S_2/S_1, 0.49), but there was no change in the basal release. CPA in doses ranging from 0.1 to 10 μM decreased the electrically evoked [^{3}H]-acetylcholine release in a concentration-dependent manner (Table 1).

DPCPX, a selective A_1 adenosine receptor an-

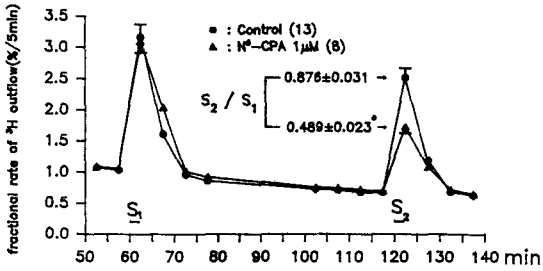

Fig. 1. Effect of N^6-Cyclopentyladenosine(N^6-CPA) on the outflow of tritium from rat hippocampal slices preincubated with ^{3}H-choline. The slices were electrically stimulated twice for 2 min each, after 60 and 120 min of superfusion(S_1, S_2). The drug effects on the stimulation-evoked outflow of tritium are expressed by the ratio S_2/S_1. Asterisks indicate the significant difference between groups($p<0.001$). The radioactivity of the tissue at the start of experiments are 0.982±0.052(control) and 1.136±0.131(N^6-CPA group) pmol. N^6-CPA was pretreated 15 min before S_2. The means±SEMs of experiments(n) are given.

Table 1. Effect of N^6-Cyclopentyladenosine(CPA) on the electrecally-evoked and basal outflow of tritium from rat hippocampal slices preincubated with ^{3}H-choline

Drugs before S_2(μM)	n	S_2/S_1	b_2/b_1
None	17	0.8361±0.0245	0.6421±0.0134
CPA 0.1	7	0.6602±0.0288*	0.6336±0.0303
0.3	4	0.5201±0.0493*	0.6076±0.0122
1.0	8	0.4892±0.0218*	0.6620±0.0347
3.0	5	0.3697±0.0509*	0.6046±0.0375
10.0	5	0.3590±0.0479*	0.5737±0.0190

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 drug-free control are marked with asterisks(*=$p<0.01$).

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

Drugs at S_2(μM)	n	S_2/S_1	b_2/b_1
Control	17	0.8361±0.0245	0.6421±0.0134
DPCPX 1	4	0.8399±0.0293	0.7043±0.0323
2	7	0.8699±0.0253	0.7100±0.0188*
5	4	0.9231±0.0367	0.7032±0.0334
10	4	0.9803±0.0060**	0.6672±0.0524

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.

tagonist (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 CPA and DPCPX, the effects of CPA 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 CPA on DPCPX-treated slices as compared with those of non-treated group. The decrements of tritium-outflow were significantly inhibited by DPCPX and the dose-response curve were shifted to the right.

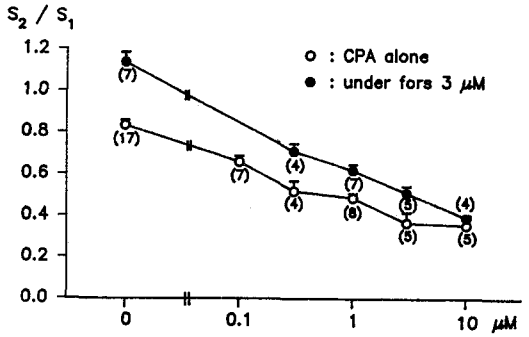

Fig. 2. Influence of 8-Cyclopentyl-1, 3-dipropylxanthine(DPCPX) on the effect of N^6-CPA on the electrically evoked tritium-outflow from rat hippocampus. Each point denotes mean±SEM from 5 to 17 experiments, but the SEM smaller than the width of the points are not shown. Asterisks indicate the significant difference between both groups(*=p<0.01, **=p<0.001). Other legends are the same as in Fig. 1.

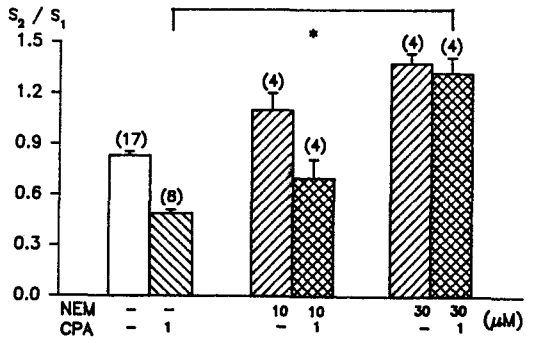

Fig. 3. Influence of the N-Ethylmaleimide(NEM) on the effect of N^6-CPA on the electrically evoked tritium outflow from rat hippocampus. NEM was added to the medium between the two stimulations for 30 min. In parentheses are the number of experiments. Asterisk indicates the significant difference(p<0.001) between the NEM-free and NEM-treated groups. Other legends are the same as in previous figures.

Effect of N-ethylmaleimide (NEM) on [^{3}H]-acetylcholine release

In order to study whether the CPA effects are mediated by G-protein, the effects of CPA were examined in the presence of the NEM, a SH-alkylating agent. NEM (10 & 30 μM) increased the tritium-outflow in a dose-dependent manner. The decreaments of tritium-outflow by 1 μM CPA were completely abolished by pretreatment with 30 μM NEM (Fig. 3).

Interactions of forskolin and CPA on [^{3}H]-acetylcholine release

As shown in Table 3, forskolin, an activator of adenylate cyclase (Seamon *et al.*, 1983), in doses ranging from 0.3 to 10 μM, increased the electrically evoked [^{3}H]-acetylcholine release in a dose-related fashion, but there was no change in the basal rate of release.

To ascertain the interaction between forskolin and CPA, the effects of CPA were studied in the presence of forskolin. Forskolin was infused from

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

Drugs at S_2(μM)		n	S_2/S_1	b_2/b_1
Control		9	0.8959±0.0281	0.7161±0.0267
Fors	0.3	11	0.8935±0.0197	0.7258±0.0159
	1.0	4	1.0271±0.0497*	0.6987±0.0252
	3.0	5	1.1714±0.0576*	0.7128±0.0329
	10.0	5	1.2736±0.0528*	0.7138±0.0283

Forskolin(Fors) added 30 min before S_2. Other legends are the same as in Table 1.

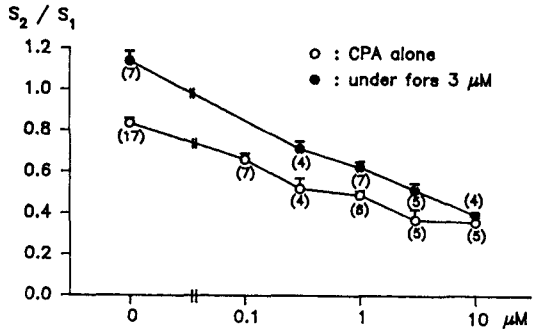

Fig. 4. Influence of forskolin(fors) on the effect of N^6-CPA on the electrically evoked tritium outflow from rat hippocampus. In parentheses are the number of experiments. Other legends are the same as in previous figures.

30 min before S_2, with CPA added at 15 min later. As shown in Fig. 4, in the presence of 3 μM forskolin, the CPA dose-dependently decreased the evoked tritium-outflow in similar fashion to those without forskolin.

DISCUSSION

Effects of N^6-Cyclopentyladenosine and related drugs on [^{3}H]-ACh release

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 actions on neuronal excitement (Phillis and Wu, 1981; Dunwiddie, 1985). Two adenosine receptors, termed A_1-and A_2-subtype, have been differentiated based on the pharmacological profiles (Daly *et al.*, 1983; Hamprecht and Van Calker, 1985; Williams, 1989), and both subtypes of adenosine receptor were found to exist in the rat hippocampus (Fredholm *et al.*, 1986b). Inhibiton by adenosine of release of neurotransmitters including acetylcholine, norepinephrine and glutamate in the hippocampus has been reported, and the presynaptic receptor participated in inhibitory effect of adenosine was defined as A_1-subtype (Jakisch *et al.*, 1983, 1984; Jonzon and Fredholm, 1984; Fredholm *et al.*, 1986a).

In the present study, the electrically evoked secretion of [^{3}H]-ACh from rat hippocampal slices was inhibited by N^6-cyclopentyladenosine, a selective A_1-receptor agonist. This result is in accordance with other reports that the R-N^6-(2-phenylisopropyl)adenosine, an adenosine analogue, decreased the electrically evoked release of acetylcholine in rabbit hippocampus (Jackisch *et al.*, 1984) and also in rat hippocampus (Duner-Engström and Fredholm, 1988).

Moreover, 8-cyclopentyl-1, 3-dipropylxanthine, a selective A_1-receptor antagonist, increased the electrically evoked tritium-outflow and inhibited the effect of CPA. These facts indicate that A_1-adenosine receptors play an important role in ACh release in rat hippocampus.

On the other hand, it has been repeatedly established that effects of A_1-adenosine receptor coupled to adenylate cyclase are mediated by guanine-nucleotide-binding protein (G protein) (Cooper *et al.*, 1980), which was defined as Gi (Katada and Ui, 1982) and can be irreversibly inhibited by the sulfhydryl alkylating agent N-ethylmaleimide (Jakobs *et al.*, 1982; Smith and Harden, 1984). Therefore, in order to confirm whether the Gi-protein is involved in A_1-receptor-mediated modulation of ACh release, the influence of NEM upon the CPA effects was investigated in this study.

When the hippocampal slices are treated with NEM, the evoked ACh release was significantly enhanced, and the CPA effects were abolished by NEM-pretreatment. It is further noted that NEM treatment significantly enhanced the basal release of [^{3}H]-ACh (0.76$\pm$0.013%/5 min, 10 μMand 0.994 $\pm$0.035%/5 min, 30 μM vs 0.642$\pm$0.013%/5 min, control), suggesting that the Gi-protein had been exerting a tonic influence upon the A_1-receptor-mediated ACh release in hippocampus. This finding agrees well with those of others who found that the various receptor-coupled inhibition of neurotransmitter release is mediated by G protein (Allgaier *et al.*, 1987; Fredholm *et al.*, 1986b; Hertting *et al.*, 1987).

Influence of forskolin upon effect of N^6-cyclopentyladenosine on [^{3}H]-ACh release

Since it was known that forskolin activates adenylate cyclase in tissue (Metzger and Lindner,

1981), the agent has been widely used as a tool for studying the involvement of cAMP in physiological events (Seamon and Daly, 1983). In the present study, the electrically evoked outflow of [^{3}H]-ACh from the hippocampal slices was enhanced by forskolin. This result is in accodance with other reports that forskolin potentiated the electrically evoked release of acetylcholine in guinea pig myenteric plexus (Alberts and Ögren, 1988), in superior cervical ganglion (Briggs *et al.*, 1988), and from hippocampal tissues of the rabbit (Choi *et al.*, 1991) and of the rat (Duner-Engström and Fredholm, 1988; Vickroy and Cadman, 1988). These facts, in conjuction with its ability to activate adenylate cyclase (Seamon *et al.*, 1983), subsequently leading to the increased intracellular cAMP (Vickroy and Cadman, 1988), indicate that the release of acetylcholine is coupled to an increase in intraneuronal cAMP.

In interaction experiments, the concentration-response relationship for CPA was observed also in the presence of forskolin. Increasing the CPA concentration overrode the acetylcholine-releasing effect of forskolin, shifting the CPA dose-response curve to the right by forskolin pretreatment. This result suggests that A_1-receptor-coupled inhibition of ACh release by CPA is closely related to the action site of forskolin.

Recently, however, Duner-Engström and Fredholm (1988) observed that effects of forskolin and rolipram (phosphodiesterase inhibitor) were completely antagonized by R-PIA, and thus, proposed that the adenylate cyclase system is not involved in A_1-receptor-regulated ACh release in rat hippocampus. Discrepancy between our result and theirs may not be easily reconciled, but one of the possibilities accounting for the difference is that they did not observe the dose-response relationships of forskolin and of CPA under forskolin pretreatment. And also there are evidence that the potassium channel (Dunwiddie, 1985; Trussell and Jackson, 1985) and calcium channel (Dolpine *et al.*, 1986) are involved in the adenosine effect. Hence, further studies are required to determine the exact post-receptor mechanisms in the ACh release mediated by presynaptic adenosine receptor in the rat hippocampus.

Overall, the results of the present study may indicate that the decrement of the evoked ACh release by CPA is mediated by the receptor-coupled inhibition of adenylate cyclase. Conclusively, endogenous cAMP may be involved in the A_1-receptor-mediated control of ACh release in the rat hippocampus.

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=국문초록=

흰쥐 해마에서 Acetylcholine 유리에 미치는 N^6-Cyclopentyladenosine 및 Forskolin의 영향

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흰쥐 해마(hippocampus)에서 acetylcholine(Ach) 유리에 미치는 A_1-adenosine 수용체의 역할과 post-receptor 기전에 있어서 adenylate cyclase 계의 관여여부에 관한 지견을 얻고자 하여 [^{3}H]-choline으로 평형시킨 해마 slice를 사용하여 [^{3}H]-ACh 유리에 미치는 여러가지 약물들의 영향을 관찰하였다.

A_1-adenosine 수용체 흥분제인 N^6-cyclopentyladenosine(CPA, 0.1～10 μM)은 전기자극(3Hz, 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, 이때 기저(basal)유리 또한 증가됨을 관찰할 수 있었고, 2 μM DPCPX 전처리는 CPA의 효과를 길항하여 CPA에 의한 용량반응곡선을 우측으로 이동시킴을 볼 수 있었다.

G protein 억제제인 N-ethylmaleimide(NEM, 10 & 30 μM)는 그 자체에 의하여 자극에 의한 ACh 유리를 증가시켰으며, 기저유리 또한 증가함을 볼 수 있었다. NEM 전처리에 의하여 CPA의 효과는 완전히 소실되었다.

한편 adenylate cyclase 활성화제인 forskolin(0.3～10 μM)은 기저유리에 변함없이 용량의존적인 [^{3}H]-ACh 유리의 증가를 초래하였으며 3 μM forskolin 전처리는 대량(10 μM)의 CPA의 효과를 제외하고는 CPA의 효과를 억제시킴을 관찰할 수 있었다.

이상의 실험 결과로 흰쥐 해마의 choline 작동성신경의 presynaptic A_1-adenosine heteroreceptor는 ACh 유리에 중요한 역할을 하고 있으며, ACh 유리의 조절에 Gi-단백질을 통한 adenylate cyclase 계의 관여가 확실하다 하겠다.