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Interaction of Forskolin with the Effect of Oxotremorine on [^{3}H]-Acetylcholine Release in Rabbit Hippocampus

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ABSTRACT

As it has been reported that the depolarization-induced release of acetylcholine(ACh) is diminished by activation of presynaptic muscarinic autoreceptor in rabbit hippocampus and various lines of evidence indicate the involvement of adenylate cyclase system in ACh release, it was attempted to delineate the role of cAMP in the muscarinic autoreceptor-mediated control of ACh release. Slices and synaptosomal preparations from rabbit hippocampus were incubated with [^{3}H]-choline and the release of the labelled products was evoked either by electrical stimulation or by high-K^+, and the influence of various agents on the evoked tritium release was investigated.

Forskolin, a specific adenylate cyclase activator, in concentrations ranging from 0.1 to 30 μM, increased the [^{3}H]-ACh release in a dose-dependent manner and also dbcAMP increased the tritium outflow. The responses to oxotremorine, a specific muscarinic agonist, were characterized by decrement of ACh release in dose range of 0.1-30 μM, and the oxotremorine effects were inhibited either by forskolin or by atropine. Glibenclamide, a specific K^+-channel inhibitor, in concentration of 1~10 μM, decreased the evoked ACh release slightly and inhibited the enhancing effect of evoked ACh-release of a large dose(10 μM) of forskolin.

These results indicate that the cAMP might play a role in the muscarinic ACh receptor-mediated control of ACh rlease in the rabbit hippocampus and suggest that certain potassium currents may also be participated in the post-receptor mechanism of ACh release.

Key Words: Hippocampus, [^{3}H]-ACh release, Forskolin, Oxotremorine, Presynaptic muscarinic autoreceptor

INTRODUCTION

Modulation of neurotransmitter release may occur by altering the intraneuronal Ca^{2+}-concentration and/or by regulating the activity of enzymes involved in the release mechanism. One of these enzymes, adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase, seems to participate in the depolarization-induced norepinephrine release, since adenylate cyclase activators, forskolin and fluoride, as well as phosphodiesterase inhibitors and cAMP analogues have been shown to facilitate the electrically evoked release of [^{3}H]-NE (Starke, 1987). Therefore, activation of adenylate cyclase with subsequent elevation of intracellular cAMP seems to plays a crucial role in neurotransmitter release. However, the contention that the adenylate cyclase system play a key role also in acetylcholine (ACh) release from neural tissues (Alberts and Ögren, 1988; Briggs *et al.*, 1988; Duner-Engström and Fredholm, 1988) has been put into question by the recent observation the

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forskolin directly reduces certain K^+-currents in addition to its action on adenylate cyclase (Hoshi *et al.*, 1988).

On the other hand, the electrically evoked secretion of ACh from cholinergic nerve is regulated by presynaptic muscarinic ACh receptor in several tissues (Stjärne, 1975; MacIntosh and Collier, 1976; Vizi, 1979; Alberts, 1982). Although activation of muscarinic ACh receptors has been shown to affect cellular levels of cAMP and of cGMP (Jakobs *et al.*, 1981; Tjörnhammer and Bartfai, 1984) and involvement of Ca^{2+}ions (Albert *et al.*, 1985; Hedlund *et al.*, 1985), the involvement of cAMP in the control of ACh release by the presynaptic muscarinic receptors has not been clearly elucidated yet.

The present study, therefore, was designed to clarify the role of cAMP in the evoked ACh release and its possible interaction with muscarinic ACh receptor-mediated control of ^{3}H-ACh release in the rabbit hippocampus.

METHODS

Tritium outlow from rabbit hippocampal slices preincubated with [^{3}H]-choline

Slices (400 μm; 4~6 mg) were prepared from the hippocampus of rabbit (1.8~2.5 kg, either sex) with a Balzers tissue chopper and were incubated in 2 ml of modified Krebs-Henseleit medium (Table 1) containing 0.1 μmol/l [^{3}H]-choline for 30 min at 37°C. Subsequently, the [^{3}H]-choline-pretreated slices were superfused with medium containing hemicholinium-3 (10 μM) for 140 min at a rate of 1 ml/min. The superfusate was continuously aerated with 95% O_2+5% CO_2 with the pH adjusted to 7.4. Colleciton 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). In cases of K^+ stimulations, the K^+ concentration of the perfusate was raised from 6 to 30 mM. The increase in [K^+] was compensated by an equipmolar decrease in [Na^+]. 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.

Tritium outflow from rabbit hippocampal synaptosomes

Synaptosomes of rabbit hippocampus were prepared as described by Choi *et al.* (personal communication). One hippocampus was homogenized with tissue homogenizer (Potters) in ice-cold 0.32 M sucrose and centrifuged for 10 min at 1,000 g and the supernatant was further centrifuged at 15,000 g for 10 min. The resulting crude mitochondrial fraction (P_2) was incubated for 10 min at 37°C in 4 ml of Krebs-Henseleit buffer (Table 1) containing [^{3}H]-choline. After the incubation, the suspension was centrifuged at 1,000 g for 10 min and the pellets were resuspended with medium to 400 μl and adjusted to a protein con-

Table 1. Composition of the incubation and superfusion medium

Components(mM)	Slice		Synaptosome
	Electric stimulation	KCl stimulation	
NaCl	118.00	118.00	121.00
KCl	4.80	4.80	1.80
$CaCl_2$	2.50	1.20	–
KH_2PO_4	1.20	1.20	1.20
$MgSo_4$	1.20	1.20	1.20
$NaHCO_3$	25.00	25.00	25.00
glucose	11.00	11.00	11.00
ascorbic acid	0.57	0.57	0.57
Na_2EDTA	0.03	0.03	–

centration of 2.2 mg/ml. Subsequently, the [^{3}H]-choline pretreated synatosomes were superfused with the medium containing hemicholinium-3 (10 μM) for 90 min at a rate of 0.8 ml/min. Collection of 5-min fraction of superfusate (4 ml) began after 40 min of superfusion. After 60 min of superfusion, 3-min stimulation with 30 mM KCl and 100 μM Ca^{2+} was performed. Drugs were added before S. At end of supefusion, the tissue were solubilized in 1% Triton X-100, and the radioactivity were counted.

Tritium determination of the superfusates and solubilized tissues was made by liquid scintillation counting (Beckman LS 5000TD). The fracitonal rate of tritium outflow (5 min^{-1}) was calculated as tritium outflow per 5 min divided by the total tritium content in the slice or the synaptosomes at the start of the respective 5 min period (Hertting *et al.*, 1980). As shown previously, the electrical stimulation of brain slices incubated with [^{3}H]-choline causes the release of only [^{3}H]-acetylcholine (Richardson and Szerb, 1974). Durg effects on the evoked tritium outflow were evaluated by calculating the ratio of the outflow evoked by S_2 and the outflow evoked by S_1(S_2/S_1).

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

The following chemicals were used: [methyl-^{3}H]-choline chloride (72~78 Ci $mmol^{-1}$, Amersham), forskolin (RBI), oxotremorine HCl (Sigma), atropine sulfate (Sigma), and Hemicholinium-3 (Sigma). Drugs were dissolved in the medium except for forskolin which was initially dissolved in DMSO and then diluted in the medium.

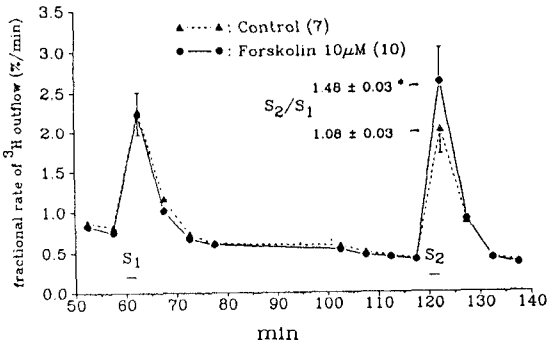

Fig. 1. Effect of forskolin on the outflow of tritium from hippocampal slices preincubated with [^{3}H]-choline. The slices were electrically stimulated twice for 2 min each, after 60 and 120 min of superfusion (S_1, S_2). Effects of drug on the stimulation-evocked outflow of tritium are expressed by 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 the experiments are 1.604 ±0.194 (control) and 1.687±0.158 (forskolin group) pmol. Forskolin was pretreated 30 min before S_2. The means±SEM of experiments (n) are given.

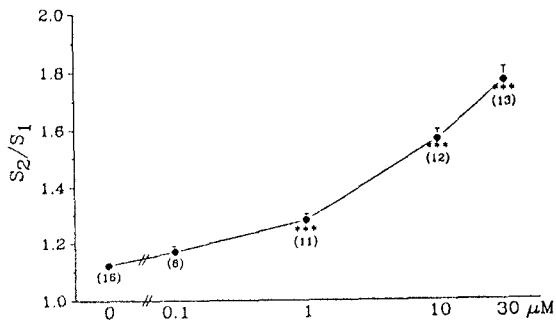

Fig. 2. Effect of forskolin on the electrically evoked tritium outflow from hippocampal slices. Means±SEM are given. In parentheses are the number of experiments. Statistically significant difference from drug-free control was marked with asteriskes (***=$p<0.001$). Other legends are as in Fig. 1.

RESULTS

Effect of forskolin and oxotremorine 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). During superfusion, the tissue was electrically stimulated twice.

As shown in Figure 1, 10 μM forskolin, an activator of adenylate cyclase (Seamon *et al.*,

1983), increased the electrically evoked outflow of tritium (S_2/S_1, 1.48), but there was no change in basal release. Forskolin in dose ranging from 0.1 to 30 μM, increased the electrically evoked [^{3}H]-acetylcholine release in a concentration-dependent manner (Fig. 2). Oxotremorine, a specific M_2-agonist, decreased the evoked tritium outflow in a dose-dependent fashion without any change of basal rlease (Table 2).

To ascertain the interaction between forskolin and oxotremorine, the effects of oxotremorine were investigated in the presence of the forskolin. Both drugs were added to the superfusion medium between S_1 and S_2 (forskolin at 30 min, oxotremorine at 15 min before S_2). In presence of forskolin (10, 30 μM), the oxotremorine decreased the evoked tritium outflow in a dose-related fashion and the effects were similar to those without forskolin (Fig. 3).

Effect of various agents on [^{3}H]-acetylcholine release from hippocampal synaptosomes

Hippocampal synaptosomes preincubated with [^{3}H]-choline were superfused with the medium containing hemicholinium-3 (10 μM). During superfusion the tissue was stimulated with 30 mM K^+ and 100 μM Ca^{2+}.

Under these experimental conditions, the ACh contents in the supernatant and tissue were determined by an enzymatic liquid-cation exchange method (Nemeth and Cooper, 1979). In these assays, 74% (n=5) of the radioactivity of supernatants and the tissue was found to be [^{3}H]-acetylcholine.

Oxotremorine decreased the K^+-evoked tritium outflow in a dose-dependent fashion and atropine abolished the oxotremorine effect. Forskolin potentiated the K^+-evoked tritium outflow but the magnitude of potentiation was less than those found in case of slice experiment, and forskolin attenuated the oxotremorine effect (Fig. 4, Table 3).

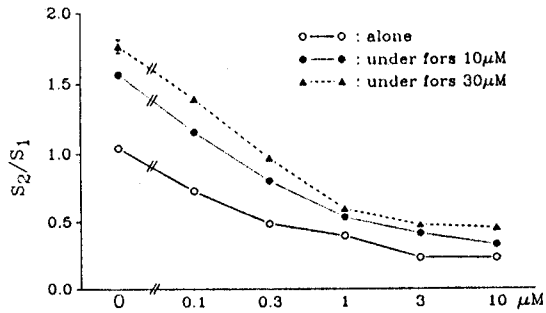

Fig. 3. Influence of forskolin on the effect of oxotremorine on the electrically-evoked tritium outflow from rabbit hippocampus. Oxotremorine pretreated 15 min before S_2. Each point denotes means±SEM from 5 to 19 experiments, but the SEMs smaller than the with of the points are not shown.

Table 2. Effect of oxotremorine(Oxo) on the electrically-evoked and basal outflow of tritium from rabbit hippocampal slices preincubated with ^{3}H-choline

Drug at S_2(μM)	% of control		n
	S_2/S_1	b_2/b_1	
control	100.0±1.92	100.0±3.70	19
Oxo 0.1	70.2±1.92*	101.9±3.70	8
0.3	47.1±2.88*	105.6±3.70	8
1.0	28.8±1.92*	96.3±1.85	7
3.0	22.1±0.96*	98.1±1.85	9
10.0	22.1±2.88*	111.1±1.85*	6

After preincubation, the slices was superfused with medium containing hemicholinium-3 (10 μM) and stimulated twice (S_1, S_2). Drugs were present from 15 min before S_2 onwards in the concentration indicated. Drug effect on basal outflow are expressed as ratio b_2/b_1 between fractional rate of outflow immediatly before S_2 (95~100 min) and the fractional rate of outflow immediatly before S_1 (55~60 min). Means±SEM of number (n) of observations are given. Significant differences from drug-free control were marked with asterisks(*=$p<0.001$).

Table 3. The effects of oxotremorine, atropine and forskolin on the 30 mM KCl evocked tritium overflow from rabbit hippocampal synaptosomes loaded with ^{3}H-choline

Drugs	μM	S/b (% of control)	n
None		100.0±3.2	9
Oxotremorine	0.3	95.7±3.0	5
	1	89.6±2.2*	5
	3	79.5±2.2***	7
	10	71.7±3.4***	8
	30	77.9±1.3***	12
Atropine	2	80.1±1.3	5
Atropine + Oxotremorine	2 10	82.7±2.3	8
Forskolin	10	116.8±2.8*	5
	30	118.2±4.7*	5
Forskolin + Oxotremorine	30 10	90.6±3.5	5

** (Oxotremorine 10 vs. Atropine + Oxotremorine); ** (Oxotremorine 10 vs. Forskolin + Oxotremorine)

After preincubation, the synaptosomes were superfused and were stimulated (S). Drug effect is expressed as ratio s/b between fractional rates of stimulation (60~65 min). Significant differences are marked with asterisks (*=p< 0.05, **=p<0.01, ***=<0.001). Other legends are as in previous tables and figures.

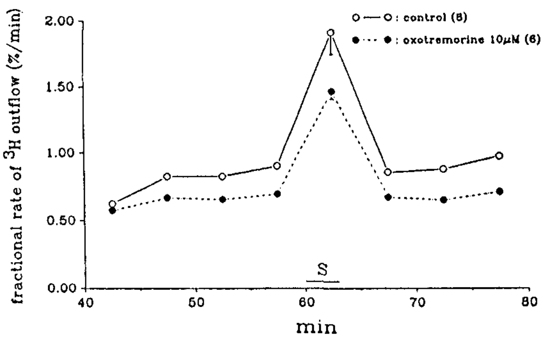

Fig. 4. After preincubation with [^{3}H]-choline, the synaptosomes were superfused with the medium containing hemicholinium-3 (10μM) and stimulated (S) for 3 min. Oxotremorine was added at 15 min before S. The radioactivity of the tissue at the start of the experimens was 0.271±0.012 pmol (n=14).

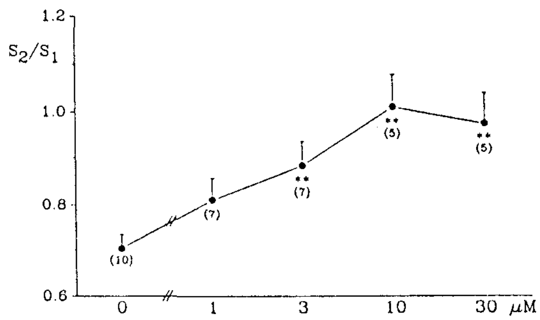

Fig. 5. Effect of forskolin on the 30 mM KCl-evoked tritium outflow from hippocampal slices. Statistical significant difference from drug-free control(0) was marked with asterisks(**=p< 0.01). Other legends are as in Fig. 1.

Effect of various agents on [^{3}H]-acetylcholine release evoked by K^+ stimulation

Slices prelabelled with [^{3}H]-choline, were superfused with the medium and were stimulated with 30 mM KCl twice. Forskolin increased K^+-evoked tritium outflow in a dose-dependent fashion (Fig. 5), and also dbcAMP increased the K^+-evoked tritium outflow. Interaction of

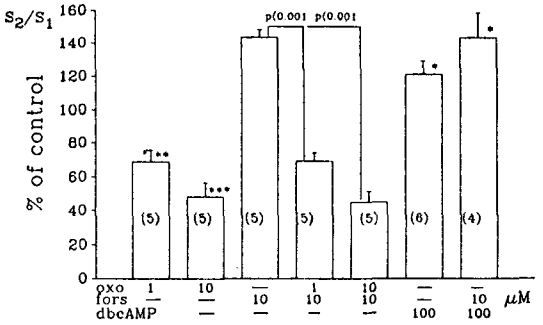

Fig. 6. Interaction between oxotremorine (oxo), forskolin (fors) and dbcAMP on the K^+-evoked outflow of tritium from hippocampal slices preincubated with [^{3}H]-choline. The K^+ concentration was raised to 30 mM twice for 2 min each, after 60 and 120 min of superfusion (S_1, S_2). Drugs added between S_1 and S_2 are indicated below the columns. Other legends are as in previous figures.

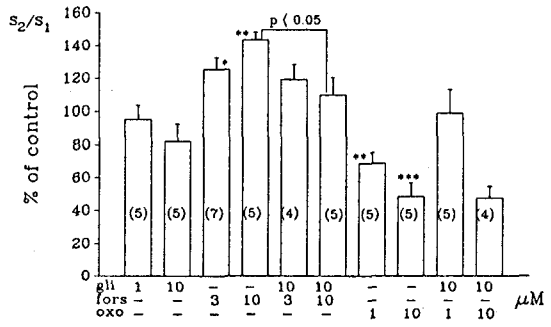

Fig. 7. Interaction between glibenclamide (gli), forskolin and oxotremorine on the K^+-evoked outflow of tritium. Other legends are in Fig. 6.

forskolin with oxotremorine and dibutyryl cyclic AMP (dbcAMP) was then studied. As shown in Fig. 6, when the slices were treated with combination of forskolin (30 min before S_2) and oxotremorine (15 min before S_2), oxotremorine completely inhibited the potentiating effects of forskolin. The dbcAMP increased outflow of tritium. When the slices were treated with combination of dbcAMP and forskolin, no additive effects were noted.

In order to investigate whether the K^+- currents were involved or not in the tritium outflow in these experiments, the influence of glibenclamide, a specific k^+-channel inhibitor (Schmidt-Antomarchi *et al.*, 1987), upon the effects of forskolin and oxotremorine was examined (Fig. 7). Glibenclamide (1, 10 μM) inhibited the tritium outflow slightly, but the effect was not statistically significant. Glibenclamide 10 μM did not alter the effect of 3 μM forskolin but inhibited the effect of 10 μM forskolin. The effect of oxotremorine was not affected by glibenclamide.

DISCUSSION

Effect of forskolin on [^{3}H]-ACh release

Since it was known that the forskolin, a naturally occurring diterpene, activates adenylate cyclase in tissue as well as in broken cell preparations (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 experiments, the electrically evoked secretion of [^{3}H]-ACh from the hippocampal slice was concentration-dependently enhanced by forskolin. This result is in accordance 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 in rat hippocampus (Duner-Engström) and Fredholm, 1988). These facts, in conjunction with its specificity to adenylate cyclase (Seamon *et al.*, 1983), suggest that the release of acetylcholine is coupled to an increase in intraneuronal cAMP.

Recently, however, it has been reported that forskolin directly reduces certain K^+-currents in addition to its action on adenylate cyclase (Hoshi *et al.*, 1988). In addition, Allgaier *et al.* (1990) observed that the 1,9-dioxy forskolin which fails to activate adenylate cyclase (Seamon *et al.*, 1983) increased the ACh release, thus, they proposed that the altered gating of voltage-dependent potassium currents may be the major underlying mechanism of ACh release by forskolin in hippocampus.

On the other hand, in guinea pig myenteric plexus, a role of neuronal cAMP in acetylcholine release is indicated by the fact that 8-Br-cAMP

(Alberts and Ögrane, 1988) increases ACh release. However, in hippocampus 8-Br-cAMP did not affect the ACh release (Allgaier *et al.*, 1990). In the present study, 8-Br-cAMP failed to affect the evoked [^{3}H]-ACh release (data not shown), but dbcAMP did increase the release. Furthermore, the high potassium-evoked secrection of [^{3}H]ACh from the hippocampal slice was concentration-dependently enhanced by forskolin. Discrepancies between the present results and the above reports, may not be easily reconciled. But among many possibilities differences in tissues employed as well as in drug specificity may be responsible.

There are, however, several points not to be overlooked in the action of forskolin. The maximal response elicited by forskolin in high potassium stimulation experiments, was less than that in the electrically evoked [^{3}H]-ACh release. Besides, the increments of [^{3}H]-ACh release by forskolin were not dose-dependent in synaptosomal preparations, and the magnitudes of the effects were far less than those in slice experiment. Also, the effects of larger doses of forskolin were inhibited by glibenclamide.

Overall, the above observations suggest that the increased [^{3}H]-ACh release by forskolin in rabbit hippocampus is related to a great extent to the increased intraneuronal cAMP and that certain potassium currents may also be involved to a lesser extent in the forskolin effect.

Effect of oxotremorine and forskolin on [^{3}H]-ACh release

It is well known that the the activation of presynaptic muscarinic ACh receptors leads to reduction of ACh secrection. And the presynaptic muscarinic receptor in the rabbit hippocampus was defined as M_2-subtype (Strittmater *et al.*, 1982). Activation of the M_2-receptors was found to influence the levels of cyclic nucleotides and the drugs that increase cAMP levels enhance [^{3}H]-ACh release (Jakobs *et al.*, 1981).

The present experiment showed that the oxotremorine, a specific M_2-subtype agonist (Messer *et al.*, 1989), decreased the electrically evoked [^{3}H]-ACh release in a dose-related fashion and that the oxotremorine effect was abolished by atropine. In interaction experiments, the concentration-response relationship for oxotremorine was observed in the presence of forskolin. Oxotremorine significantly inhibited the ACh releasing effect of the forskolin. These results suggest that the M_2 receptor-coupled inhibition of ACh release by oxotremorine is closely related to the action site of forskolin.

It is now widely accepted that the receptor-coupled inhibition of adenylate cyclase and signal transduction mechanism of presynaptic α_2-autoreceptors is mediated by regulatory guanine-nucleotide-binding protein G_i (Rodbell, 1980; Cooper, 1982; Allgaier *et al.*, 1986; Fredholm *et al.*, 1987). More recently, Hertting *et al.*, (1988) observed that the N-ethylmaleimide(NEM), a specific G protein alkylating agent (Jakobs *et al.*, 1984), auguments the electrically evoked [^{3}H]-ACh release in rabbit hippocampus and they proposed that muscarinic auto-receptors operate the ACh release via a G-protein. Choi (personal communication, 1991) observed that NEM increased the evoked [^{3}H]-ACh release and also inhibited the decreasing action of oxotremorine on the ACh release.

On the basis of these findings, the results of the present study may indicate that the decrements of the evoked ACh release by oxotremorine is mediated by the receptor-coupled inhibition of adenylate cyclase. Conclusively, endogenous cAMP may be involved in the muscarinic ACh receptor-mediated control of ACh release in the rabbit hippocampus.

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= 국문초록 =

가토 해마에서 Acetylcholine 유리에 미치는 Oxotremorine 및 Forskolin의 영향

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가토 해마(hippocampus)에서 acetylcholine(ACh) 유리에 미치는 cAMP의 역할에 관한 지견을 얻고자하여 [^{3}H]-choline으로 평형시킨 해마 slice 및 시냅토솜(synaptosome)을 사용하여 [^{3}H]-ACh 유리에 미치는 여러가지 약물들의 영향을 관찰하였다.

Adenylate cyclase 활성화제인 forskolin(0.1～30 μM)은 전기 및 고농도 K^+ 자극에 의한 [^{3}H]-ACh 유리를 용량 의존적으로 증가시켰으며, dbcAMP 또한 ACh 유리를 강화시켰다. Muscarine성 흥분제인 oxotremorine(0.1～30 μM)은 전기 및 K^+ 자극에 의한 [^{3}H]-ACh 유리 효과를 용량 의존적으로 감소시켰으며, 이러한 효과는 forskolin 및 atropine에 의하여 차단됨을 관찰할 수 있었다.

한편 K^+-channel 억제제인 glibenclamide(1, 10 μM)는 자체로는 K^+ 자극에 의한 [^{3}H]-ACh 유리를 약간 억제시킴을 관찰할 수 있었으며, 대량의 forskolin(10 μM)에 의한 [^{3}H]-ACh의 증가 효과를 감약시킴을 알 수 있었다.

이상의 실험 결과로 가토 해마의 presynaptic muscarinic autoreceptor를 통한 ACh 유리의 조절에는 세포내 cAMP의 관여가 확실하다 하겠으나, 일부 K^+-currents의 관여를 배제할 수는 없을 것으로 사료된다.