Arch. Pharm. Res. Vol.19, No.6, pp. 520-528, 1996

Effects of Forskolin on Endogenous Dopamine and Acetylcholine Release in Rat Neostriatal Slices

Hwa-Jung Kim

College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea

(Received September 13, 1996)

The involvement of the cyclic AMP (cAMP) effector system in the release of endogenous dopamine and acetylcholine from the rat neostriatum was assessed. Forskolin, an activator of adenylate cyclase, was used to enhance cAMP production, and the consequence of this enhancement on the spontaneous and potassium stimulated release of dopamine and acetylcholine was evaluated. Neostriatal slices were prepared from Fischer 344 rats and after a preincubation period the release of each endogenous neurotransmitter was measured from the same slice preparation. To measure acetylcholine release the slice acetylcholinesterase (AChE) activity was inhibited with physostigmine, but the release from slices with intact AChE activity was also determined (choline, instead of acetylcholine was detected in the medium). Under both conditions forskolin induced a significant dose-dependent increase in the potassium-evoked release of dopamine. In the same tissue preparations the release of neither acetylcholine (AChE inhibited) nor choline (AChE intact) was affected by forskolin. The results indicate that the cAMP second messenger system might be involved in neuronal mechanisms that enhance neostriatal dopamine release, but stimulation of this second messenger by forskolin does not further enhance neostriatal acetylcholine release.

Key words : cAMP effector system, Forskolin, Acetylcholine, Dopamine, Endogenous release, Neostriatal slices

INTRODUCTION

Forskolin, a potent activator of adenylate cyclase in many tissues, causes a rapid and reversible elevation of cyclic AMP (cAMP) and the elevation of cAMP by forskolin has been linked to a variety of cellular responses (Seamon *et al.*, 1983; Seamon *et al.*, 1981).

Although the role of cAMP in many cellular functions is now generally accepted, its involvement in the release of neurotransmitters is still not completely clear. In cholinergic neurons the enhancement of the release of acetylcholine by forskolin or various drugs increasing cAMP has been reported to occur mostly in peripheral systems including Guinea pig ileal synaptosomes (Reese and Cooper, 1984), myenteric neurons of Guinea pig small intestine (Yau *et al.*, 1987), neuromuscular junction of mouse hemidiaphragms (Dryden *et al.*, 1987) and rat superior cervical ganglion (Briggs *et al.*, 1988). In the rat neostriatum, however, it has been shown that elevation of cAMP by drugs such as 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor) and 8-bromo-cAMP (an analog of cAMP) did not evoke [^{3}H]acetylcholine release (Stoof and Kebabian, 1982).

The effects of changes in cAMP levels on the release of other neurotransmitters have also been demonstrated. In cerebrocortical brain slices, forskolin potentiated [^{3}H]-norepinephrine release (Markstein *et al.*, 1984) and cAMP derivatives potentiated stimulation-evoked catecholamine release (Tsujimoto *et al.*, 1986). Furthermore, cAMP has been reported to be a second messenger modulating tyrosine hydroxylase activity and dopamine synthesis in brain via D2 receptors (Masserano and Weiner, 1983). There is an evidence that potassium-evoked dopamine release from pheochromocytoma (PC-12) cells is potentiated by stimulation of cAMP levels (Baizer and Weiner, 1985) and stimulation of adenylate cyclase by forskolin with IBMX potentiated Ca^{2+}-evoked [^{3}H]dopamine release in striatal synaptosomes (Bowyer and Weiner, 1989). Santiago and Westerink (1990) also showed that cAMP increased the extracellular dopamine in striatum of freely-moving rats. However, Patrick and Barchas (1976) reported that the cAMP analog, dibutyryl cAMP, did not affect K^+-evoked release of dopamine from striatal synaptosomes.

Correspondence to: Hwa-Jung Kim, College of Pharmacy Ewha Womans University 11-1, Daehyun-Dong, Seodaemun-Gu Seoul, 120-750

In the majority of studies to date, radioactive precursors were used to label the tissue acetylcholine or dopamine; changes in the release of these neurotransmitters were assessed by measuring radioactive outflow and using it as an index of changes in release of the endogenous neurotransmmitters. Some investigators, however, have studied the release of endogenous acetylcholine or dopamine and the effects of pharmacological agents on the release of the endogenous neurotransmitters. Although in many cases the overflow of radiolabeled species accurately reflected the release of endogenous acetylcholine or dopamine, pools of newly synthesized neurotransmitter, i.e., radiolabeled pools, were not always affected by pharmacological agent in the same manner as endogenous pools (Beani *et. al.*, 1984; Herdon *et al.*, 1985). Precautions should be taken, therefore, when evaluating the effects of pharmacological manipulations on acetylcholine or dopamine release.

The purpose of present study was to investigate the effects of forskolin on the stimulation-evoked release of endogenous acetylcholine and dopamine from rat neostriatal slices. To test whether in the rat neostriatum the elevation of cAMP does differently affect acetylcholine release and dopamine release, the simultaneous effect of forskolin on the release of the two neurotransmitters from the same neostriatal slice was examined. That is, because of the potential interactions of the dopaminergic system with the cholinergic system (Lehmann and Langer, 1983), from the same neostriatal slice, the release of endogenous dopamine and endogenous acetylcholine was monitored. The effects of forskolin were tested both in the presence and in the absence of an acetylcholinesterase (AChE) inhibitor so acetylcholine release could be measured directly (AChE inhibited) or indirectly (AChE intact).

MATERIALS AND METHODS

Animals and brain slice preparation

Male F344 rats (3 months old) weighing 250-300 g were housed individually in suspended stainless steel cages in a room maintained at 22°C and under a 12-hour light/dark cycle.

Rats were sacrificed by decapitation, the brain was rapidly removed and slices were prepared from trimmed striatal tissue blocks using a glass guide according to the procedure described by McIlwain and Rodnight (1962). While neostriatal slices were prepared, they were continuously moistened with warmed (36°C), oxygenated (95% O_2-5% CO_2) Krebs Ringer (KR) bicarbonate solution. The slices (7-9) from each rat were then preincubated for 3 hours in oxygenated KR solution. From any given rat each slice was tested under a different experimental condition. Thus, for each value reported the sample size, n, also represents the number of rats tested under that given experimental condition.

All experiments were performed at 36°C in the KR bicarbonate buffer containing (concentrations in mM): NaCl 124; KCl 5.1; $MgSO_4$ 1.3; KH_2PO_4 1.22; $NaHCO_3$ 25.5; $CaCl_2$ 2; and glucose 10.2. The composition of the 25 mM KCl buffer was the same, except that NaCl was reduced to 104 mM to correct for osmolarity changes.

Release of endogenous acetylcholine and dopamine

The slices from each rat were equilibrated in 30-35 ml KR buffer for 3 hours, then single slices were transferred to tissue holders and incubated for 20 min in 3 ml of fresh KR buffer containing various concentrations of forskolin (1 μM, 10 μM and 50 μM) and/or other pharmacological agents. For some of the experiments, physostigmine (30 μM) was also added to the incubation media to inhibit acetylcholinesterase. For most of the experiments the control slices were exposed to 0.1% ethanol. Since 50 μM forskolin was dissolved in 0.5% ethanol, some of the experiments were done using 0.5% ethanol for all the test samples. Because there was no difference in the control responses elicited either in 0.1% or 0.5% ethanol, the values were pooled as one control.

Following this 20-min exposure period spontaneous and K^+-stimulated release were monitored during two subsequent 5-min incubations in 1 ml of regular KR buffer and 25 mM K^+-KR buffer, respectively, in the presence of the same agents mentioned above. After the K^+-depolarization, the tissues were removed from the tissue holder and homogenized to measure the tissue content of dopamine and its metabolite, dihydroxyphenylacetic acid (DOPAC), acetylcholine and choline.

Dopamine and DOPAC assay

For the dopamine and DOPAC release determination, an aliquot (200 μM) of the release media was added to ice-chilled tubes containing 0.01 nmole dihydroxybenzylamine (DHBA) as the internal standard and acidified with perchloric acid (PCA) to a final concentration of 0.4 N. The tissues were homogenized in 0.4 N PCA (1 ml) containing 0.1 nmole DHBA and 1nmole each of $^2H_9$-acetylcholine and $^2H_9$-choline (internal standards for the acetylcholine and choline determination). The tissue homogenate was set on ice for 20 min then centrifuged at 10,000×g for 20 min. One aliquot (500 μM) of the tissue supernatant was taken for the tissue determination of dopamine and DOPAC and the second 500 μM aliquot was used for the a-

cetylcholine and choline determination (see below). The pellet was dissolved in 1 N-NaOH for protein determinations. Both the release and tissue samples were stored at -80°C until analysis. Dopamine and DOPAC were quantified using High Performance Liquid Chromatography (HPLC) with electrochemical detection. Release samples were injected onto an Ultrasphere (C18) ODS column (5 μm, 4.6×105 mm, i.d.; Anspec) and tissue supernatants were injected onto a Biosphere (C18) ODS column (5 μm, 4.6×250 mm, i.d.; BAS). The mobile phases used for each column consisted of 0.1 M Citrate, 0.075 M NaHPO 4, 0.75 mM Sodium heptanesulfate and either 5-6% methanol for release samples or 10-12% methanol for tissue samples with a final pH of 3.9. The mobile phase was filtered and degassed under vacuum. All separations were performed at a flow rate of 0.9 ml/min and a detector potential of 0.71 volt. Chromatographic conditions, retention times and sensitivity were tested daily with standards before analysis of samples. The quantification of DA and DOPAC was calculated from standard curves and corrected for variable loss using peak height ratios of the dopamine or DOPAC to DHBA. The detection limit for dopamine and DOPAC was about 0.05 pmoles/50 μl.

Acetylcholine and choline assay

The acetylcholine and choline content in medium andtissue samples was assayed by GCMS according to Jenden *et al.* (1973) and Freeman *et al.* (1975). For the release samples, a 600 μl aliquot of incubation medium was added to tubes containing $^{2}H_{9}$-acetylcholine and $^{2}H_{9}$-choline (0.5 nmoles each) as internal standards and the samples were extracted at pH 9.2 (TAPS buffer) into dichloromethane containing 1 mM dipicrylamine. For the tissue samples, the tissue supernatant was neutralized with 5 N K_2CO_3 (50 μl) and set on ice for 20 min to allow for precipitation of $KClO_4$. The samples were then centrifuged for 20 min at 10,000×g; the supernatant was washed 2 times with ether, and acetylcholine and choline were ion-pair extracted with dipicrylamine in dichloromethane. After the ion-pair extraction both medium and tissue samples were analyzed similarly.

Briefly, the analysis after ion-pair extraction involvedderivatization of the choline, demethylation of the quaternary amines and liquid-liquid extraction and concentration into the organic phase. Acetylcholine and choline were quantitated relative to the deuterated internal standards by selected ion monitoring of the most abundant fragment with a Spectral Mass Spectrometer. The sensitivity of this assay is in the femtomolar range.

Dopamine-depletion with 6-hydroxydopamine

The stereotaxic surgeries, as described by Orr *et al.* (1986), were performed 5-7 days after the arrival of the rats. The rats were anesthetized with equithesin (0.25 ml/100 gm body weight), and 6-HDA (250 虜 free base) or the vehicle (0.1% ascorbic acid in 0.9% NaCl) was infused (10 μl per hemisphere; 1.5 μl/min) into the lateral ventricles at the following coordinates (Paxinos and Watson, 1986): posterior 0.6 mm, lateral 吊 1.2 mm, ventral -3.5 mm. The animals were given intraperitoneal injections of pargyline (40 mg/kg) and desmethylimipramine (DMI: 25 mg/kg) 20 to 30 minutes before the 6-HDA or vehicle infusions in the treated and control rats, respectively. Pargyline was used to potentiate the effect of 6-HDA by inhibiting MAO, and DMI was used to prevent the uptake of the neurotoxin into noradrenergic nerve terminals. Food and water consumption were monitored daily, and as the animals became aphagic and adipsic, they were injected with rice cereal (p.o.) and injected with physiological saline (i.p.). Half the control rats were also given rice and saline. From these rats neostriatal slices were prepared, as described above, five days after the stereotaxic surgery.

cAMP assay

Tissue cAMP levels were analyzed by radioimmunoassay (Amersham).

Protein assay

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard. The tissue content and release of the endogenous neurotransmitters and the cAMP content were expressed on the basis of slice protein.

Chemicals

Forskolin, tris(hydroxymetyl)-methyl-aminopropane (TAPS), DHBA, physostigmine (eserine) and BSA were obtained from Sigma (St. Louis, MO), S(-)-Sulpiride from Research Biochemicals Inc. (Natick, MA), dipicrylamine (2,2',4,4',6,6'-hexadinitrophenylamine) from Pfaltz and Bauer, Inc. (Waterbury, CT), and dichloromethane (99+%) from Aldrich (Milwaukee, WI).

The deuterated internal standards and reagents for the acetylcholine/choline assay were provided by Dr. Donald Jenden, Dept. of Pharmacology, University of California, Los Angeles.

Statistics

All results are expressed as the mean±S.E., and the sample size, n, represents the number of slices tested. Pairwise comparisons were done by the two-tailed

Student's t test.

RESULTS

In each experiment the release of acetylcholine and dopamine was monitored under two conditions-when the slice AChE activity was inhibited with physostigmine, and when the slice AChE activity was left intact. Under the former condition (AChE inhibited) both acetylcholine and choline were detected in the medium, whereas under the second conditioin (AChE intact) no acetylcholine was detected in the medium. It had been assumed that the choline measured represented, to a large extent, the acetylcholine released and hydrolyzed by the AChE (Lee *et al.*, 1991; Weiler, 1989). Under both conditions, the release of dopamine and DOPAC was monitored from the same slices that the release of acetylcholine or choline was detected.

Effect of forskolin on the release of the endogenous amines

Forskolin significantly increased the potassium-stimulated release of dopamine in a dose-dependent manner. The potassium-evoked release of dopamine from neostriatal slices with intact AChE activity was enhanced by 2-, 3- and 4.5-fold in the presence of, respectively, 1, 10 and 50 μM forskolin (Fig. 1A, open symbols). The DOPAC detected in the release samples was also increased in a dose-dependent manner by 16%, 76% ($p<0.02$), 134% ($p<0.005$) at 1, 10 and 50 μM forskolin (data not shown). The amount of DOPAC released depends on the amount of dopamine available for deamination, and a major pool of the neuronal dopamine available for deamination is supplied from reuptake of released dopamine Cubeddu *et al.*, 1979). In these same slices in which forskolin enhanced the release of dopamine, the adenylate cyclase activator had negligible effects on choline release (Fig. 1B, open symbols).

To test whether effects of forskolin on acetylcholine release would be manifest also under conditions of AChE inhibition, a similar experiment was run in the presence of physostigmine. Again forskolin significantly increased dopamine release (Fig. 1A, solid symbols) but had no apparent effect on either acetylcholine or choline release from the same slices (Fig. 1B, solid symbols).

The effect of forskolin on the spontaneous release of the amines was also monitored (Table 1). Forskolin did not significantly alter the spontaneous release of acetylcholine or choline from the neostriatal slices. For the experiments done in the absence of physostigmine a total of seven to eight spontaneous release samples were monitored for the control and each concentration of forskolin tested. In many of these samples, especially control and at lower concentrations of forskolin, the spontaneous release of dopamine was not detectable. If these values are considered as zero and are averaged with the detectable amounts in that respective group (0.0015 ± 0.0008, 0.0011 ± 0.0010, 0.0046 ± 0.0013 and 0.0055 ± 0.0016 nmoles/mg protein for control, 1 μM, 10 μM and 50 μM of forskolin, respectively), there was a dose-dependent increase in the spontaneous release of dopamine which was significant ($p<0.05$) in the presence of 50 μM forskolin only. In the presence of physostigmine and for all concentrations of forskolin tested the spontaneous release of dopamine was higher than the release from slices with intact AChE activity.

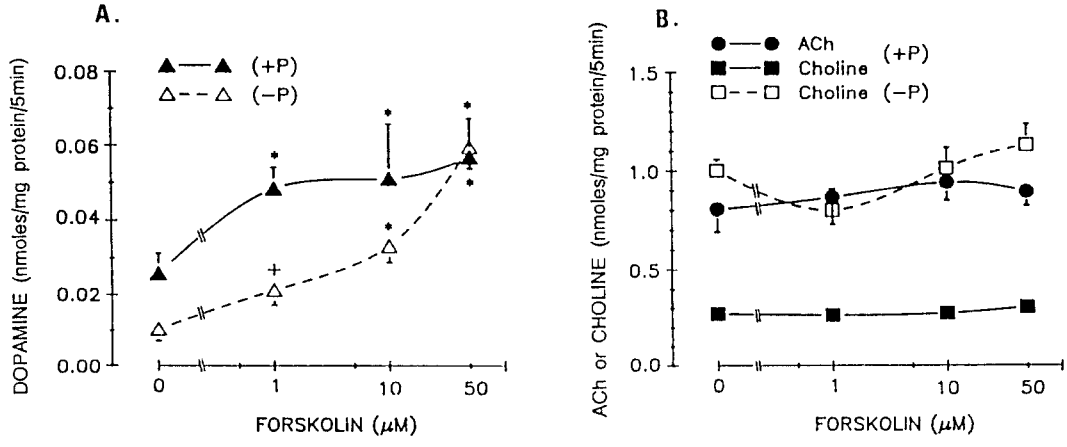

Fig. 1. Dose-dependent effects of forskolin on the potassium-evoked release of dopamine, acetylcholine, and choline from neostriatal slices of F344 rats. A. Dopamine release (nmoles/mg protein/5 min). B. Acetylcholine and choline release. The open symbols are release values obtained from slices with intact AChE activity (-P, no physostigmine). The closed symbols represent release values when AChE inhibited with physostigmine (+P). Each bar is the mean±S.E. of 4-6 independent observations. *$p<0.05$ relative to release in the absence of forskolin

This release did not vary with the increase in the forskolin concentration.

The spontaneous release of DOPAC increased with increasing concentrations of forskolin when AChE activity of the slices was intact (no physostigmine). The increases in DOPAC were 108% ($p<0.05$), 123% and 275% ($p<0.05$) in the presence of 1, 10 and 50 μM forskolin, respectively. There also was a dose-dependent increase in the amount of DOPAC in the medium in the presence of physostigmine (128% and 271% in the presence of 10 and 50 μM, respectively). But this increases in DOPAC in the presence of physostigmine were not statistically significant.

Effect of forskolin on tissue content of the amines

Overall, the tissue content of dopamine, DOPAC, acetylcholine and choline was not altered in the presence of forskolin (Table 2). Thus, the increase in dopamine and DOPAC release by forskolin was not accompanied by significant changes in the tissue content of these compounds.

Effect of forskolin in the release of the endogenous amines in dopamine-depleted slices

As summarized above, forskolin significantly enhanced the potassium-evoked release of dopamine with no apparent effect on the release of acetylcholine from the neostriatal slices. Dopamine agonists have been shown to decrease acetylcholine release in the neostriatum (Stoof and Kababian, 1982). It is possible that forskolin did induce an increase in acetylcholine neostriatal release, as has been observed in the myenteric plexus (Yau *et al.*, 1987). However, the increase in acetylcholine release by forskolin could have been counteracted by an inhibition of acetylcholine release by the endogenous do-

Table I. Effect of forskolin on spontaneous release of dopamine, DOPAC, acetylcholine and/or choline from rat neostriatal slices

AChE Intact (-P) Forskolin (μM)	Dopamine	DOPAC	Choline
0	0.004±0.001 (3)	0.008±0.002 (8)	0.288±0.076 (8)
1	0.004 (2)	0.016±0.002[a] (6)	0.310±0.089 (8)
10	0.005±0.002 (5)	0.017±0.005 (7)	0.260±0.027 (8)
50	0.007±0.001 (6)	0.029±0.004[a] (8)	0.360±0.071 (8)
AChE Inhibited (+P) Forskolin (μM)	**Dopamine**	**DOPAC**	**Acetylcholine**
0	0.025±0.003[b] (7)	0.007±0.001 (3)	0.077±0.007 (8)
1	0.029±0.006[b] (4)	0.006±0.001 (4)	0.093±0.018 (4)
10	0.027±0.008[b] (4)	0.016±0.008 (4)	0.085±0.010 (4)
50	0.029±0.007[b] (7)	0.026±0.004 (7)	0.103±0.015 (8)

Each value is the mean±S.E. of the number of independent observations indicated in parentheses and expressed in nmoles/mg protein/5 min. The release of neurotransmitters was tested either in the absence of physostigmine (-P) or in the presence of physostigmine (+P). The spontaneous release of choline in the presence of physostigmine (AChE inhibited) was also not significantly altered in the presence of forskolin (not shown).

[a] $p<0.05$ relative to the control release in the absence of forskolin

[b] $p<0.01$ relative to the release at the same concentration in the absence of physostigmine

Table II. Tissue content of dopamine, DOPAC, acetylcholine and choline in neostriatal slices

Forskolin (μM)	Dopamine	DOPAC	Acetylcholine	Choline
AChE Intact				
0	1.329±0.137	0.212±0.033	3.071±0.225	1.704±0.114
1	1.458±0.100	0.195±0.034	1.955±0.264	1.955±0.264
10	1.538±0.154	0.301±0.028	3.322±0.212	1.659±0.072
50	1.396±0.121	0.301±0.024[a]	3.489±0.330	1.874±0.184
AChE Inhibited				
0	1.720±0.135	0.373±0.031	4.520±0.390	1.046±0.134
1	1.707±0.240	0.316±0.049	3.970±0.740	1.277±0.121
10	2.023±0.098	0.475±0.034	4.238±0.315	1.313±0.077
50	1.433±0.081	0.333±0.020	4.300±0.036	1.291±0.109

Each value is the mean±S.E. of four to eight independent observations and expressed in nmole/mg protein.

[a] $p<0.05$ relative to the control release in the absence of forskolin

pamine interacting with receptors on the cholinergic neurons. To examine this possibility, i.e., that the dopamine increased in the presence of forskolin was depressing release from the cholinergic neurons, the effects of forskolin were examined under two different conditions: in slices depleted of dopamine by in vivo 6-hydroxydopamine treatment and in intact slices in the presence of sulpiride, a D2 antagonist.

In slices depleted of 95% of their tissue dopamine (Table 3), the release of dopamine averaged 4% of that released from control slices. Forskolin had no effect on the release of this remaining pool of dopamine. The tissue DOPAC was also decreased (35% of control) by the 6-hydroxydopamine treatment, and this metabolite was not detected in the medium of the dopamine-depleted slices. The 6-hydroxydopamine treatment had no apparent effect on the cholinergic neurons in the neostriatum. The tissue content of acetylcholine and choline was unchanged and potassium-stimulated release was similar to that observed in the control slices (Table 3).

Not shown in Table 3 are the results of another dopamine depletion experiment. In this second experiment, 6-hydroxydopamine treatment resulted in only a 36% depletion of dopamine, and the release of dopamine from the 6-hydroxydopamine slices averaged 59% that of control. In these slices the effects of forskolin on release were monitored in the presence of physostigmine. Forskolin increased dopamine release by 33% (10 μM) and 25% (50 μM; $p<0.058$) in these slices partially depleted of dopamine. Acetylcholine release in the presence of 0, 10 and 50 μM forskolin was, respectively, 1.21±0.17 (n=4), 1.24±0.11 (n=3), and 1.27±0.26 (n=4) nmoles/mg protein/5 min. Thus, when acetylcholine release was measured directly, forskolin did not significantly affect acetylcholine release even when there was a partial depletion of the dopamine pools.

Table III. Tissue content of the amines and DOPAC in vehicle-treated (Control) and 6-hydroxydopamine-treated (6-HDA) slices-effects of forskolin on choline release

Tissue Content	Control	6-HDA
Dopamine	1.356±0.077	0.068±0.006*
DOPAC	0.095±0.019	0.033±0.010*
Acetylcholine	3.265±0.264	2.679±0.308
Choline	2.046±0.233	1.500±0.133
Release of Choline	**Control**	**6-HDA**
No Drug	1.159	1.230
Forskolin 10 μM	1.256	0.985
Forskolin 50 μM	1.244	1.069

Each tissue value is the overall mean±S.E. of five to six slices from control and 6-HDA-treated rats. The effects of forskolin were tested in two slices at each concentration, and the value given is the average from the two slices at that forskolin concentration. Each value is expressed in nmoles/mg protein.
*$p<0.05$ relative to the control

Effects of forskolin on the release of the amines in the presence of the dopamine D2 antagonist, sulpiride

Another way to attenuate the possible inhibitory action of dopamine on the cholinergic neurons is to block its interaction with dopamine D2 receptors on these neurons (Lehman and Langer, 1983). Sulpiride is an effective D2 antagonist that will block the inhibitory effects of dopamine agonists on neostriatal acetylcholine release (Cubeddu and Hoffman, 1983), so the effects of forskolin were examined in the presence of this dopamine antagonist. These results are summarized in Fig. 2.

Again, as in the experiments summarized above, forskolin (50 μM) significantly enhanced the release of dopamine (2.8-fold, $p<0.05$), but had minimal effects on the release of choline in slices with intact AChE activity (Fig. 2A). Sulpiride (1 μM) alone enhanced the release of dopamine by 91%, and when combined with forskolin, release was similar to that in the presence of forskolin alone. Choline release from these same slices was slightly increased (30%) by sulpiride alone, but neither forskolin nor the combination of forskolin and sulpiride had any effect on choline release.

In this same experiment the AChE activity of a group of slices was inhibited with physostigmine, and the release of dopamine was about 2.5 fold greater (0.

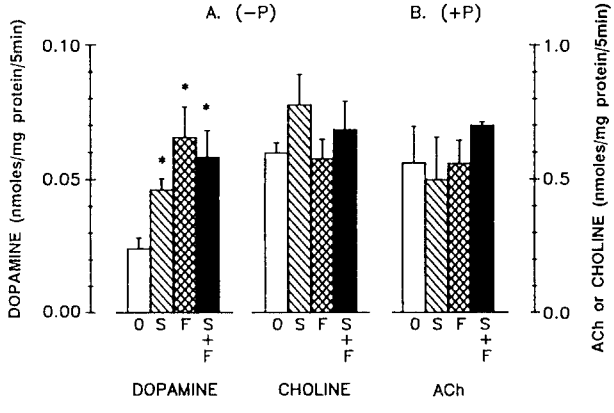

Fig. 2. Effects of forskolin (50 μM), sulpiride (1 μM), and the combination on potassium-evoked release of dopamine, acetylcholine and choline from rat neostriatal slices. A. Dopamine and choline release from slices with intact AChE activity (-P). B. Acetylcholine release from slices with AChE activity inhibited with physostigmine (+P). Each bar graph is the mean±S.E. of 3-4 independent observations, and the values are nmoles/mg protein/5 min release period. Control (open bars), sulpiride (diagonal bars), forskolin (hatched bars), combination (solid bars).
*$p<0.05$ relative to the no drug control

063±0.013 nmoles/mg protein/5 min) than that released in the absence of physostigmine. The magnitude of the increase in dopamine release by sulpiride (33%) or forskolin (50%) was not as great as that observed in the slices with intact AChE activity. Together, sulpiride and forskolin induced a 84% ($p<0.054$) increase in dopamine release. Acetylcholine release from these slices was not significantly affected by any of the drug treatments, either alone or in combination (Fig. 2B).

Effects of forskolin on the slice cAMP content

The effects of increasing concentrations of forskolin on cAMP formation in neostriatal slices were measured in the absence of phosphodiesterase inhibitors. Forskolin induced a dose-dependent increase in the cAMP levels in slices with both intact and inhibited AChE activity (Fig. 3A). There was an apparent leftward shift in the forskolin dose-response curve when the slice AChE activity was inhibited with physostigmine. In dopamine-depleted slices forskolin also significantly enhanced cAMP formation (Fig. 3B), but the maximal response elicited by forskolin in these slices was about 27% less than that elicited in the control slices. Physostigmine treatment also induced a leftward shift in the forskolin dose-response curve of these dopamine-depleted slices.

In the sulpiride experiments the forskolin (50 μM)-induced increase in the control slice cAMP levels (from 18±2 to 297±20 pmoles/mg protein; n=4) was not significantly altered when sulpiride was also in the preparation (227±48 pmoles cAMP/mg protein; n =4). This was also true for those slices exposed to physostigmine (cAMP in control: 15±2; forskolin: 261±68; forskolin plus sulpiride: 235±52 pmoles/mg protein; all n=4). Sulpiride alone also did not significantly affect the slice cAMP levels (15±2 and 11±1 in the control and physostigmine-exposed slices, respectively).

DISCUSSION

In the present study, forskolin was used to raise intracellular levels of cAMP to evaluate the role of the cAMP effector system on neostriatal acetylcholine and dopamine release. Dose-response curves for forskolin were generated and ability of forskolin to stimulate cAMP production was confirmed at the experimental condition (slice preparation) used in this study. The results show that forskolin induced a significant dose-related stimulatory effect on potassium-evoked dopamine release from neostriatal slices prepared from 3-month F344 rats, but its effects on acetylcholine release were negligible. This indicates that the cAMP effector system enhanced by forskolin is involved in the release of dopamine from dopaminergic nerve endings in the neostriatum.

The results of this study also show that stimulation of the cAMP effector system by forskolin does not further enhance neostriatal acetylcholine release. This implies that either neostriatal acetylcholine release is not enhanced by the cAMP effector system or that acetylcholine release is already maximal when slices are depolarized with 25 mM otassium. It is possible that amplification of the effector system will not further amplify the cholinergic release esponse. In regard to the latter alternative, previous sudies have shown that depolarization of the neostriatal slices with 25

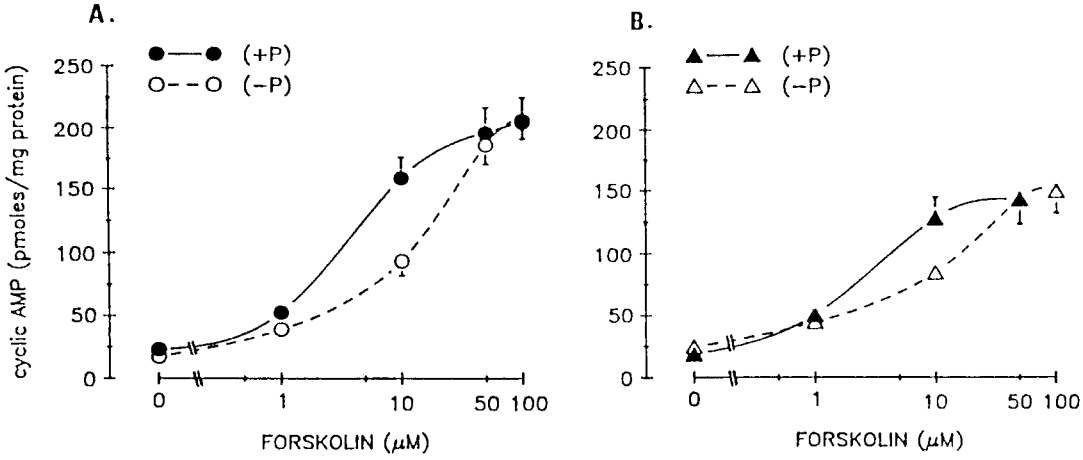

Fig. 3. Dose-dependent effects of forskolin on cAMP production in rat neostriatal slices. A. Cyclic AMP levels in control neostriatal slices when AChE is intact (open symbols) and inhibited (solid symbols). Each value represents the mean±S.E. of at least 4 independent observations (range 4-9). B. Cyclic AMP levels in dopamine depleted slices (neostriatal slices were prepared from 6-hydroxydopamine-treated rats) when AChE is intact (open symbols) and inhibited (solid symbols). Missing error bars are within the symbols given. Relative to the control, forskolin significantly enhanced cAMP production ($p<0.05$) at all concentrations in all the groups examined, so the asterisks were not included in the figure.

mM potassium does not induce maximal release of either acetylcholine or dopamine (Lee *et al.*, 1991). Thus, acetylcholine release from the slice preparation can be enhanced by other means (higher potassium concentrations). The observation that forskolin did not affect neostriatal acetylcholine release is in contrast to results seen in peripheral cholinergic systems (see introduction), but it is supported by a previous study in which it was shown that agents such as IBMX and 8-bromo-cAMP, which enhance and mimic cAMP, respectively, had no effect on acetylcholine release in the rat neostriatum (Stoof and Kababian, 1982). The results of this present study might not imply that cAMP is not involved in neostriatal acetylcholine release. Agents which decrease cAMP formation were not tested for their concomitant effects on the forskolin stimulated cAMP formation and the release of acetylcholine and dopamine.

Because of the potential for dopaminergic inhibition of the cholinergic neurons in the slice preparation, the effects of forskolin were also examined in dopamine-depleted slices and in the presence of the D2 antagonist, sulpiride. Again, there were no significant changes in the release of acetylcholine and choline in the presence of forskolin. Thus, it is unlikely that the dopamine release increased by forskolin was counteracting any effect of forskolin on the cholinergic neurons. The assumption made with the sulpiride experiments was that the concentration of sulpiride tested was effectively inhibiting any action of dopamine on D2 receptors. This seems to be a reasonable assumption based on reports in which sulpiride was used to inhibit the action of D2 agonists on acetylcholine release (Stoof and Kababian, 1982). Also, the sulpiride was effectively inhibiting the D2 autoreceptors because it alone induced an increase in dopamine release (Fig. 2A).

It is possible that the release of other neurotransmitters was affected by forskolin, and these neurotransmitters were counteracting effects of forskolin on acetylcholine release. For example, gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the neostriatum. It is unlikely, however, that GABA would be counteracting possible effects of forskolin on acetylcholine release since the release of GABA is attenuated by forskolin (Weiss, 1988).

When acetylcholine release was monitored directly in the medium by inhibiting AChE with physostigmine, this manipulation had significant effects on dopamine release. That is, in the presence of physostigmine, dopamine release increased significantly, as observed in previous studies (Lee *et al.*, 1991; Lehman and Langer, 1982), and the slices were more sensitive to the effects of forskolin on dopamine release (Fig. 1A). Whether this effect of physostigmine on dopamine release is mediated by acetylcholine remains to be tested, although Lehman and Langer (1982) observed that the muscarinic antagonist, atropine, blocked the increase in dopamine release due to physostigmine exposure.

In summary, the enhancement of dopamine release by forskolin implies that the cAMP effector system could be involved in mechanisms that increase neostriatal dopamine release. Acetylcholine release did not increase in the presence of forskolin, and it might be possible that whereas the cAMP system could be involved in mechanisms to decrease acetylcholine release (e.g., autoinhibition by muscarinic receptors), other effector mechanisms could be involved in the enhancement of neostriatal acetylcholine release. This will be examined by testing other agents that stimulate other second messenger systems for their concomitant effects on neostriatal acetylcholine and dopamine release.

ACKNOWLEDGEMENT

The author greatly appreciate Dr. Molly S. Weiler, Hazleton Wisconsin Inc., for invaluable discussion and critical review of manuscript, and thank Dr. Donald J. Jenden, Dept. of Pharmacology, University of California Los Angeles, for the use of the GCMS facilities for the acetylcholine and choline determinations. This study was in part supported by faculty research fund from Ewha Womans University.

REFERENCES CITED

Baizer, L. and Weiner, N., Nerve growth factor treatment enhances nicotine-stimulated dopamine release and increases in cyclic adenosine 3':5'-monophosphate levels in PC 12 cell cultures. *J. Neurosci.*, 5, 1176-179 (1985).

Beani, L., Bianchi, C., Siniscalchi, A., Sivilotti, L., Tanganelli, S. and Veratti, E., Different approaches to study acetylcholine release: endogenous ACh verses tritium efflux. *Naunyn Schmiedeberg's Arch. Pharmacol.*, 328, 119-126 (1984).

Bowyer, J.F. and Weiner, N., K^+-channel and adenylate cyclase involvement in regulation of Ca^{++}-evoked release of [^{3}H]dopamine from synaptosomes. *J. Pharmacol. Exp. Ther.*, 248, 514-520 (1989).

Briggs, C.A., McAfee, D.A. and McCaman, R.E., Long-term regulation of synaptic acetylcholine release and nicotinic transmission. *Br. J. Pharmacol.*, 93, 399-411 (1988).

Cubeddu, L.X. and Hoffman, I.S., Frequency-dependent release of acetylcholine and dopamine from rabbit striatum: its modulation by dopaminergic receptors. *J. Neurochem.*, 41, 94-101

(1983).

Dryden, W.F., Singh, Y.N., Gordon, T. and Lazarenko, G., Pharmacological elevation of cAMP and transmitter release at the mouse neuromuscular junction. *Can. J. Physiol. Pharmacol.*, 66, 207-212 (1987).

Freeman, J.J., Choi, R.L. and Jenden, D.J., Plasma choline, its turnover and exchange with brain choline. *J. Neurochem.*, 24, 729-734 (1975).

Herdon, H., Strupish, J. and Nahorski, S.R., Differences between the release of radiolabelled and endogenous dopamine from superfused rat brain slices: Effects of depolizing stimuli, amphetamine and synthesis inhibition. *Brain Res.*, 348: 309-320 (1985)

Jenden, D.J., Roch, M. and Booth, R., Simultaneous measurement of endogenous and deuterium labeled tracer variants of choline and acetylcholine in subpicomole quantities by gas chromatography mass spectrometry. *Anal. Biochem.*, 55, 438-448 (1973).

Lee, H.-J., Alcorn, L.M. and Weiler, M.H., Effects of various experimental manipulation on neostriatal acetylcholine and dopamine release. *Neurochemical Res.*, 16, 875-883 (1991).

Lehman, J. and Langer, S.Z., Muscarinic receptors on dopamine terminals in the cat caudate nucleus: Neuromodulation of [^{3}H]dopamine release in vitro by endogenous acetylcholine. *Brain Res.*, 248, 61-69 (1982).

Lehman, J. and Langer, S.Z., The striatal cholinergic interaction: synaptic target of dopaminergic terminals? *Neurosci.*, 10, 1105-1120 (1983).

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin reagent. *J. Biol. Chem.*, 193, 265-275 (1951).

Markstein, R., Digges, K., Marshall, N.R. and Starke, K., Forskolin and the release of noradrenaline in cerebrocortical slices. *Naunyn Schmeideberg's Arch. Pharmacol.*, 325, 17-24 (1984).

Masserano, J.M. and Weiner, N., Tyrosine hydroxylase regulation in the central nervous system. *Mol. Cell. Biochem.*, 53/54, 129-152 (1983).

McIlwain, H. and Rodnight, R., Preparing neural tissues for metabolic study *in vitro*, In McIlwain, H. (Eds.), *Practical Neurochemistry*. Churchill, London, pp. 109-188, 1962.

Patrick, R.L. and Barchas, J.D., Dopamine synthesis in rat brain striatal synaptosomes. II. Dibutyryl cyclic adenosine 3':5'-monophosphoric acid and 6-methyltetrahydropteridine-induced synthesis increase without an increase in endogenous dopamine release. *J. Pharmacol. Exp. Ther.* 197, 97-104 (1976).

Reese, J.H. and Cooper, J.R., Stimulation of acetylcholine release from Guinea-pig ileal synaptosomes by cyclic nucleotides and forskolin. Biochemical Pharmacol., 33, 3007-3011, (1984).

Seamon, K.B. and Daly, J.W., Forskolin, cyclic AMP and cellular physiology. *Trends Pharmacol. Sci.*, 4, 120-123 (1983).

Seamon, K.B., Padgett, W. and Daly, J.W., Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. USA*, 78, 3363-3367 (1981).

Stoof, J.C. and Kebabian, K.W., Independent *in vitro* regulation by D2-dopamine receptor of dopamine-stimulated efflux of cAMP and K^+-stimulated release of acetylcholine from rat neostriatum. *Brain Res.*, 250, 263-270 (1982).

Tsujimoto, A., Morita, K., Kitayama, S. and Dohi, T., Facilitation of acetylcholine-evoked CA release by cAMP on isolated perfused dog adrenal glands. *Arch. Int. Pharmacodyn. Ther.*, 279, 304-313 (1986).

Weiler, M.H., Muscarinic modulation of endogenous acetylcholine release in rat neostriatal slices. J. Pharm.Exp. Ther. 250, 617-623 (1989)

Weiss, S., Forskolin attenuates the evoked release of [^{3}H]GABA from s striatal neurons in primary culture. *Brain Res.*, 463, 182-186 (1988).

Yau, W.M., Dorsett, J.A. and Youther, M.L.. Stimulation of acetylcholine release from myenteric neurons of Guinea pig small intestine by forskolin. *J. Pharmacol. Exp. Ther.*, 243, 507-510 (1987).