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Effect of Bufalin on Acetylcholine Levels in Rat Brain: Relation to Its CNS-Stimulating Action

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1. Total acetylcholine (ACh) levels in the rat whole brain homogenate decreased during the maximal tonic extension induced by bufalin.

2. "Free" ACh levels in the rat whole brain homogenate increased during the maximal tonic extension induced by bufalin, but not by pentylenetetrazol.

3. ACh release from rat cerebral cortical slices increased in a medium containing bufalin.

4. After intracerebral Hemicholinium-3 or scopolamine, 50% convulsive dose of bufalin in mice was increased, but was decreased after eserine.

5. A relationship between the convulsant effect of bufalin and its ACh-releasing effect was discussed.

Keywords——bufalin; total acetylcholine levels in the brain; free acetylcholine levels in the brain; acetylcholine release from the cerebral cortical slices; convulsion

Bufalin is a cardiotonic steroid with a six-membered lactone ring extracted from Ch' an Su (Senso in Japanese), the dried venom of a Chinese native toad. It was already reported that the majority of cardiotonic steroids extracted from Ch' an Su have a stimulating effect on the central nervous system (CNS) and produce typical tonic convulsions in toxic doses.[2–4)]

In our previous work,[5)] bufalin was estimated to increase the transmitter release at the neuromuscular junction of an isolated rat diaphragm.

The present study was undertaken to verify whether or not the increase in acetylcholine (ACh) release takes place not only in the motor nerve terminal but also in the brain, and if the increase does occur, whether or not the development of a convulsion is involved in this phenomenon.

Materials and Methods

Extraction of Total ACh from Rat Brain[6)]——Male Wistar rats weighing approximately 250 g were used. Bufalin was injected at a dose of 0.8 mg/kg into the femoral vein and the animals were decapitated during the tonic extension. The head was frozen by immersion in liquid nitrogen for 5 min. The brain was chipped out of the frozen head taking care to prevent any pieces from thawing. This was pulverized using a stainless steel pestle and mortar containing liquid nitrogen.

Total ACh was extracted as follows: After adjusting 3 ml of physiological saline solution containing 15 μg/ml of eserine sulfate to pH 4.0 by adding 1 ml of acetate buffer, an accurately weighed brain powder (300—500 mg) was added to this solution and the mixture was boiled for 2 min in order to liberate the bound ACh completely. When cooled, the solution was centrifuged at 3000 rpm for 7—10 min at room temperature and the supernatant fluid was stored at $-10°$ until assay.

Extraction of "Free" ACh from Rat Brain——Male Wistar rats weighing approximately 250 g were used. "Free" ACh was extracted by the method similar to that described by Crossland and Slater.[7)] The animal

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was sacrificed by decapitation during the maximal tonic extension induced by bufalin or pentylenetetrazol in a dose of 0.8 or 40 mg/kg, *i.v.*, respectively. Excised brain without cerebellum was homogenized for 3 min with a stainless steel blade rotating at 1200 rpm in ice cold physiological saline containing 15 μg/ml of eserine sulfate and 17 μg/ml of $CuCl_2$. The volume of the medium used was 5 ml/g wet weight of the brain. The homogenate was then centrifuged and the supernatant fluid was stored at 0°. The assay was made within 2 hr.

ACh Release from Rat Cerebral Cortical Slices——Male Wistar rats weighing approximately 300 g were used. The cerebral cortical slices of 0.4 mm thick were made with a blade and guide as described by McIlwain.[8] Approximately 150 mg of slices were weighed accurately and incubated for 1 hr at 37° in 2.5 ml of the Krebs-Hensleit solution of the following composition (in mM): NaCl, 118.5; $NaHCO_3$, 24.9; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose, 10; eserine sulfate, 15 (μg/ml); and gassing with a mixture of 5% CO_2 in O_2 throughout. After incubation, the solution was stored at 10° until assay.

Assay of ACh——Male guinea pigs weighing 200—300 g were sacrificed by a blow on the head and the ileum was removed. The ileum was cut into small segments (2—3 cm long) and suspended in 2 ml of oxygenated Tyrode solution of the following composition (in mM): NaCl, 128; KCl, 2.6; $CaCl_2$, 10.5 (10 times the normal concentration); $NaHCO_3$, 11.9; NaH_2PO_4, 0.41; glucose, 5.5; morphine hydrochloride, 5 (mg/ml); tryptamine hydrochloride, 40 (mg/ml); and diphenhydramine hydrochloride, 2×10^{-8} (g/ml) at 22—25°. The contractile response of the ileum was recorded on a smoked kymograph with a semi-isotonic lever.

The supernatant fluid from the brain homogenate or the fluid incubated with the brain slice was divided equally and one was adjusted to pH 11 with 0.5 or 1 N NaOH and boiled for 2—3 min to break down ACh and neutralized with 0.5 N HCl. The unhydrolyzed supernatant fluid was diluted with saline until the concentration was appropriate for the assay while the hydrolyzed solution was diluted with a standard solution of ACh to the same degree to prevent the effect of "sensitizing substance."[6] The muscle preparation was exposed to the extract solution or the standard solution for exactly 30 sec and contractile height was recorded. Between successive determinations of ACh, the muscle left to stand for 45—90 sec. The assay was carried out by the 3-point method. Statistical analysis was carried out by the Student's t-test.

Influences on Convulsion Thresholds of Convulsants——Male ddY mice weighing approximately 20 g were used. Bufalin dissolved at a concentration of 2 mg/ml in 50% ethanol in water was injected into the caudal vein. Pentylenetetrazol and strychnine nitrate were injected *i.p.* as an aqueous solution in a concentration of 0.3 and 0.006%, respectively. Animals injected these convulsants were placed in a compartment cage at room temperature (20—23°) and observed for 1 hr, and the number of animals exhibiting tonic convulsions was counted. From these results, 50% convulsive dose (CD_{50}) was calculated by Litchfield-Wilcoxon's method.[9]

The effect on these convulsion thresholds was tested by the following drugs: Hemicholinium-3 (HC-3) injected intracerebrally 15 min before the convulsants by the method of Haley and McCormick,[10] and eserine sulfate and scopolamine hydrobromide given intraperitoneally 5 min and 20 min before convulsants, respectively. These intervals of the pretreatment were determined as the peak of the combined effect in a preliminary study.

Drugs——ACh (acetylcholine chloride, Ovisot, Daiichi), tryptamine hydrochloride (Tokyo Kasei), morphine hydrochloride (Sankyo), diphenhydramine hydrochloride (Taisho), HC-3 (Hemicholinium-3, Aldrich), pentylenetetrazol (Cardiazol, Sankyo), strychnine nitrate (Sanko Seiyaku), eserine sulfate (Tokyo Kasei), scopolamine hydrobromide (E. Merck).

Bufalin was kindly provided by Drs. M. Komatsu and Y. Kamano, Division of Organic Chemistry, Taisho Pharmaceutical Co.

Results

Effect of Bufalin on ACh Levels in Rat Brain

1) Total ACh Levels——As shown in Table I, mean total ACh levels in the whole brain homogenate caused 22% decrease during the maximal tonic extension induced by bufalin (0.8 mg/kg, *i.v.*). This increase in ACh levels was significant ($p<0.01$).

2) "Free" ACh Levels——As shown Table I, mean "free" ACh levels in the whole brain homogenate caused 70% increase during the maximal tonic extension induced by 0.8 mg/kg, *i.v.*, of bufalin. This increase in ACh levels was significant ($p<0.01$).

On the other hand, "free" ACh levels were not significantly different from those of control during the maximal tonic extension induced by 40 mg/kg, *i.v.*, of pentylenetetrazol.

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Table I. Acetylcholine Levels in the Rat Brain during the Tonic Extension Induced by Bufalin or Pentylenetetrazol

Treatment	No. of expt.	Dose (mg/kg, *i.v.*)	ACh[a]	Change (%)	Significance (p)
Total acetylcholine levels					
Control	10	—	1.57 ± 0.11	—	—
Bufalin	9	0.8	1.22 ± 0.10	−22	<0.05
Free acetylcholine levels					
Control	9	—	0.71 ± 0.11	—	—
Bufalin	7	0.8	1.2 ± 0.15	+70	<0.01
Control	7	—	0.60 ± 0.11	—	—
Pentylenetetrazol	9	40.0	0.57 ± 0.13	−5	N.S.

a) Values represent mean ± standard error, expressed as μg (chloride)/g (fresh tissue).
N.S.: not significant

Effect of Bufalin on ACh Release from Rat Cerebral Cortical Slices

As is apparent from Table II, mean ACh release from rat cerebral cortical slices caused 50% increase in a medium containing bufalin in a concentration of 8×10^{-6} g/ml. The concentration of bufalin used here approximated the plasma bufalin concentration sufficient to produce the tonic extension. This increase in ACh release is significant ($p<0.01$).

Table II. Acetylcholine Release from Rat Cerebral Cortical Slices with and without Treatment with Bufalin

Treatment	No. of expt.	Concn. (g/ml)	ACh released[a]	Change (%)	Significant (p)
Control	10	—	1.6 ± 0.20	—	—
Bufalin	10	8×10^{-6}	3.2 ± 0.37	+50	<0.01

a) Values represent mean±standard error, expressed as μg (chloride)/g (fresh tissue)/60 min.

Influence of Some Drugs on Convulsion Thresholds of Bufalin and Other Convulsants

CD_{50} value of bufalin was increased by pretreatment with intracerebral HC-3 (5 μg/animal) and scopolamine (50 mg/kg, *i.p.*) to 1.62 times and 1.71 times that of the control value, respectively. These changes in CD_{50} value were significant ($p<0.05$) by the Litchfield-

Table III. Influence of Some Drugs on Convulsion Thresholds of Bufalin and Other Convulsants (male ddY-mice)

Drugs tested[a]	Convulsants[b]	Body weight	No. of mice	CD_{50} (mg/kg) with drugs	No. of mice	CD_{50} (mg/kg) without drugs
HC-3	Buf.	18—20	25	[c]1.05(0.91— 1.21)	25	0.65(0.59— 0.72)
	Pen.	18—20	21	66.5(55.0 —80.5)	21	63.0(50.4 —78.8)
	Str.	17—22	20	1.30(1.15— 1.47)	20	1.45(1.20— 1.74)
Eserine	Buf.	22—24	21	[c]0.36(0.34— 0.38)	21	0.56(0.49— 0.64)
	Pen.	17—18	20	65.0(50.0 —84.5)	20	59.0(53.6 —64.9)
	Str.	18—20	29	1.46(1.29— 1.65)	37	1.57(1.50— 1.65)
Scopolamine	Buf.	22—24	20	[c]0.96(0.89— 1.04)	21	0.56(0.49— 0.64)
	Pen.	17—18	20	[c]67.0(62.6 —71.5)	20	59.0(53.6 —64.9)
	Str.	17—22	20	1.51(1.42— 1.60)	20	1.45(1.20— 1.74)

a) HC-3, 5 μg/animal, intracerebral injection, 15 min before convulsants
eserine sulfate, 0.25 mg/kg, *i.p.*, 5 min before convulsants
scopolamine hydrobromide, 50 mg/kg, *i.p.*, 20 min before convulsants.
b) Buf.=bufalin (*i.v.*), Pen.=pentylenetetrazol (*i.p.*), Str.=strychnine nitrate (*i.p.*)
c) significant ($p<0.05$)

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Wilcoxon method. CD_{50} value of bufalin was significantly decreased by pretreatment with eserine (0.25 mg/kg, *i.p.*).

In contrast, the convulsion threshold of pentylenetetrazol was not significantly influenced by HC-3 or eserine, but was significantly increased by scopolamine ($p<0.05$).

The convulsion threshold of strychnine was not altered significantly by HC-3, eserine, or scopolamine.

Discussion

It is well known that cardiotonic steroids generally have a convulsant effect. In the present study, the total ACh levels in rat brain was reduced significantly during the tonic extension induced by bufalin. The result obtained here agrees with those of other convulsants reported by Stater.[11]

The so-called "free" ACh levels increased significantly during the maximal tonic extension induced by bufalin but not by pentylenetetrazol. Compared with the results of other investigators,[7] the present determination of "free" ACh levels in the brain was larger to some extent. ACh levels in the brain are expected to vary considerably according to the mode of animal sacrifice, method of bioassay, *etc.* Moreover, it is assumed that "free" ACh almost always contains a certain amount of an artifact ACh which is mechanically liberated from the bound form during the period of tissue homogenization. Thus, relative but not absolute values of free ACh levels were thought to be significant.

As mentioned previously,[12] bufalin has no effect on the acetylcholineesterase activity in a concentration of 10^{-5} g/ml *in vitro*. The anticholinesterase agent is generally thought to increase both free and total ACh levels in the brain through the preservation of ACh released.[7] Bufalin differs from the anticholinesterase agent with regard to the effect on the total ACh level. Accordingly, increase in the free ACh levels induced by bufalin appears to be due to its ability to accelerate the rate of liberation of free ACh from the bound form.

Since there are no method established for the accurate extraction of free ACh at present, an *in vitro* study of ACh release was added. ACh release from rat cerebral cortical slices increased significantly in a medium containing bufalin. The concentration of bufalin used here approximated the plasma bufalin concentration sufficient to produce the tonic extension. This finding also suggests that bufalin increases the liberation of free ACh from the bound form in the brain during convulsions.

The convulsion threshold of bufalin rose significantly in mice pretreated with intracerebral HC-3. In contrast, the convulsion threshold of strychnine or pentylenetetrazol was not affected by HC-3. It has been reported that HC-3 has an inhibiting effect on both the release and synthesis of ACh in the rat brain.[11] Thus, these facts suggest that the mechanism by which HC-3 inhibits the release and synthesis of ACh antagonizes the tonic extension induced by bufalin. The convulsant action of strychnine is considered to be related to the blockade of postsynaptic inhibition.[13] The stimulant action of pentylenetetrazol may be due to a decrease in neuronal recovery time.[13] In the present experiments, "free" ACh levels were not affected during the convulsions caused by pentylenetetrazol.

The convulsion threshold of bufalin fell significantly in animals pretreated with a subconvulsive dose of eserine. As mentioned above, it has been observed that eserine increases free and total ACh in the brain.[7] Thus, the possibility is advanced that the potentiation of bufalin-induced convulsion by eserine is related to the synergism of these two compounds by which the increase in the free ACh levels in the brain is accelerated.

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The convulsion threshold of bufalin rose significantly in animals pretreated with scopolamine. Scopolamine is known to increase the ACh release in the cat brain,[14] and atropine and scopolamine do not appreciably affect the free ACh level in the rat brain.[7] Hence the antagonism of scopolamine against the bufalin-induced convulsion is not explained in terms of the ACh release or the free ACh level.

Recently, Beld, *et al.*[15] suggested that muscarinic receptors in the CNS is quite similar to those in the peripheral nervous system in pharmacological nature. Based on their view, there is a possibility that the anticonvulsant effect of scopolamine is due to its competitive antagonism against the convulsant effect of ACh released by bufalin at the muscarinic receptors in the CNS.

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