ACUTE TOXICITY OF ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE¹⁾

Minoru WATANABE, Harumi ARIKAWA,²⁾ Shigeki MIYAKE,³⁾ and Yutaka KASUYA⁴⁾

Department of Toxicology and Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-Ku, Tokyo, Japan [113]

Summary.....Acute toxicity of Na-adenosine 3',5'-cyclic monophosphate (Na-cAMP) was studied in mice. LD₅₀ values (in g/kg) in males (m.) and females (f.) by three routes were as follows : p. o. : m. 14.3, f. 15.2 ; i. p. : m. 1.14, f. 0.395 ; i. v. : m. 1.57, f. 0.645. After a depression following the Na-cAMP administration (i. p. and i. v.) wore off, a delayed, progressive severe ataxia (just like a curare-induced ataxia) was noted 10-30 hr after the administration especially in females. If the ataxia once appeared, animals died in 2-3 hr without exception, seemingly from respiratory paralysis. The females given with highest doses and most of males died earlier in an extreme depression. In the ataxic animals, the twitch tension development of the hind limb induced by sciatic nerve stimulation *in situ* was strongly suppressed as compared with that induced by direct stimulation. On the basis of the results and also of those of some electrophysiological studies *in vitro*, it is suggested that some disturbance of the motor nerve terminals may be a main cause of the ataxia. The question whether this ataxia is the primary toxic effect of cAMP or a secondary effect is now under investigation.

Whereas a large number of studies have been made on physiological functions of adenosine 3',5'-cyclic monophosphate (cAMP), only a brief description is available as regards the toxicity of cAMP in mammals (Henion and Sutherland, 1967). Since cAMP is an endogenous substance, excess exogenous cAMP will be expected to cause excessive function of some physiological processes which might result in an intoxication of an individual. Thereby two factors will be important for the appearance of toxicity of cAMP ; one is that cAMP plays important and diverse roles as a second messenger in almost all the cells in the body, and the other is that cAMP hardly, if not at all, permeates the cell membrane. These opposing factors make it difficult to foresee what will happen as lethal response to excess exogenous cAMP, and how it will be. In the present investigations, the acute toxicity of cAMP was determined in terms of LD₅₀. A characteristic delayed symptom, so to say curare-like, skeletal muscle paralysis was thereby observed preceeding

- 1) This work was presented in parts to the 47th Annual Meeting of the Japanese Pharmacological Society in April, 1974 and to the 96th Annual Meeting of the Pharmaceutical Society of Japan in April, 1976.
- 2) Present address : Department of Forensic Medicine, Faculty of Medicine, Showa University, Tokyo
- 3) Present address : Research Center, Sankyo Co., Tokyo.
- 4) Prerent adress : Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokyo,

the death. Since this delayed ataxia was an almost only toxic symptom which seemed to relate to death, we explored the site and cause of toxic dysfunction of the motor system.

METHODS & MATERIALS

Acute toxicity. Mice of both sexes of dd-strain weighing 17 to 20 g were used. They were housed in groups of 10 at 21–22°C. Sodium salt of cAMP was injected as approximately isotonic solution (56 mg/ml). For oral administration, solutions of required concentrations up to 700 mg/ml were used. The pH of these solutions was about 7.0. Animals were observed for at least 72 hr after the administration of cAMP. LD 50 values (72 hr) and their fiducial limits ($p < 0.05$) were calculated by the method of Litchfield and Wilcoxon (1949). Doses are expressed as sodium salt.

Twitch tension of the mouse hind limb in situ. Mice were anesthetized with urethane-chloralose and fixed on the ventral position. The left sciatic nerve was exposed and ligated. Bipolar platinum electrodes were set on the nerve rostral to the site of ligation for indirect stimulation. Two stainless steel needle electrodes were inserted into the middle part of the thigh for direct stimulation. The limb was freed from fixation and a toe was connected to a force displacement transducer with a silver wire of 0.05 mm thick. Rectangular pulses of supramaximal voltage at 1 Hz and of 0.1 and 1.0 msec durations were used for the indirect and the direct stimulations, respectively. Several pulses were applied every 10 min. The responses were recorded on a polygraph (Nihon-Koden, model RM-150). When d-tubocurarine, 0.5 mg/kg, was injected intraperitoneally to test the validity of this preparation, responses to indirect stimulations gradually disappeared within 45 min, while those to direct stimulations remained almost unchanged.

Conduction ability of the sciatic nerve. The conventional sucrose gap method was used to record the action potential of the sciatic nerve of the female mouse. The full length of the sciatic nerve was isolated including the part lying in the vertebra, set on the apparatus, superfused with Tyrode's solution aerated with 95 % O₂ and 5 % CO₂ at 37°C, and was stimulated with rectangular pulses of supramaximal voltage of 0.01 msec in duration. The conduction velocity was calculated from the distance between the stimulating and the recording electrodes and the time interval between the stimulation and the peak of action potential. Action potentials were displayed on a double beam cathode ray oscilloscope (Nihon-Koden, model VC-6) through a negative capacitance cathode follower (Nihon-Koden, model MZ-3).

Electrophysiological parameters of neuromuscular transmission. End-plate potentials (e. p. p.'s) and miniature end-plate potentials (m. e. p. p.'s) were recorded in vitro from phrenic nerve-diaphragm and sciatic nerve-gastrocnemius preparations isolated from normal and cAMP-intoxicated ataxic female mice (660 mg/kg of NA-cAMP, i. p.). These preparations were isolated from animals treated with cAMP when the ataxia became evident (about 1 hr after the appearance of the ataxia). Preparations were set in Tyrode's solution and aerated at 36°C with 95 % O₂ and 5 % CO₂. Nerves were stimulated through a

Acute toxicity of cyclic AMP

platinum suction electrode (rectangular pulses of 0.1 msec in duration, and of supra-maximal voltage) and muscles were stimulated through a bipolar platinum electrode (rectangular pulses of 1 msec in duration and of supramaximal voltage). Glass-micropipettes filled with 2 M KCl and of about 50 M Ω in resistance were connected to a hand-made solid-state negative-capacitance preamplifier (2 N 5524-source follower with 741 LN). The band width was restricted to about 2 kHz when m. e. p. p.'s were recorded. The potentials were visualized on a cathode ray oscilloscope and recorded with a camera (Nihon-Koden, VC-6 and PC-2B). Approximate quantum content was calculated from the mean amplitude of m. e. p. p.'s and of e. p. p.'s. The content was not corrected against resting potentials.

The contractile force of the sciatic nerve-gastrocnemius preparation was also measured using separate preparations set in the same manner. The contractile force was visualized on the oscilloscope using a hand-made strain-gauge transducer of minimal compliance with a preamplifier (AD 504).

Acetylcholine determination. The acetylcholine content of the femoral muscles of the female mouse was determined gas-chromatographically according to the method of Maruyama and Hosoya (1975). Muscles whose cholinesterase had been inactivated by micro-wave irradiation were homogenized in 0.4 N perchloric acid with a known quantity of butyrylcholine added as an internal standard. The homogenate was centrifuged, and the resultant supernatant was washed with ether, and was added with KI-I₂ solution followed by centrifugation. The precipitate was dissolved in acetonitrile and was centrifuged after an addition of a small quantity of Amberlite CG 400. The supernatant was charged to the gas-chromatograph (Shimazu, model GC-3BF) through a pyrolyzer.

Drugs. Following drugs were used. Several lots of adenosine 3',5'-cyclic mono phosphate sodium salt. They were gifts from Seishin Pharmaceutical Co. and Wakamoto Pharmaceutical Co., to whom the authors are grateful. Adenosine, adenosine 5'-mono-phosphate disodium salt (Kohjin Co.), neostigmine bromide (Tokyo-Kasei Co.) and d-tubocurarine chloride (Amelizol, Takeda Pharmaceutical Co.).

RESULTS

Acute toxicity. LD 50 values are listed in Table 1. After oral doses death occurred

Table. 1 LD 50 values of Na-cyclic AMP in mice and their fiducial limits calculated by Litchfield-Wilcoxon's method

Route	Sex	LD 50 and Fiducial limits (p=0.05) (g/kg)	
p. o.	male	14.3	12.5 — 16.5
	female	15.2	13.9 — 16.8
i. p.	male	1.14	0.94 — 1.41
	female	0.395	0.368 — 0.423
i. v.	male	1.57	1.40 — 1.76
	female	0.645	0.577 — 0.723

within about 30 hr, and the higher the dose was, the earlier it occurred. Within 2 hr after administration of 10 g/kg of Na-cAMP, a cyanosis and a reduction of locomotion were observed. These symptoms could largely be due to severe dehydration as suggested by guzzling, marked fluid retention in the intestine and by obvious recovery by intraperitoneal isotonic glucose solution. At higher doses, the locomotor depression lasted for hours, body tone and lighting reflex were lost and the animals died after 10 to 30 hr. As a fluid retention was not always observed in these animals, it is likely that they died from some intrinsic toxicity of cAMP besides dehydration. Further detailed studies have been suspended, since the necessary amount of cAMP was too large.

LD 50 values and toxic symptoms after intraperitoneal and intravenous injections were similar. A notable sexual difference was found in the toxicity when given by these two routes. Several minutes after the injection of cAMP, the spontaneous activity of the mice was reduced. They reacted to stimuli, although somewhat slowly. This depressive state lasted for 1 to 2 hr and the animals looked thereafter almost normal, and only slightly sluggish. At highest doses, some animals, especially males, died within few hours in an extreme depression. After 10 to 30 hr, mostly after 20 hr, a progressive, severe ataxia appeared especially in females. The ataxia seemed to begin at hind limbs with slight abnormal gait. When the ataxia progressed, animals moved just like grubs or green caterpillars, actively responding to external stimuli with forelimbs and dorsal muscles; the dorsal muscles seemed to remain unparalyzed longer than limb muscles and animals seemed to remain quite conscious even when they could only twist their body and could no longer even creep. The animals finally lost righting reflex and died 2 to 3 hr after the appearance of ataxia, seemingly from respiratory arrest. So long as could be confirmed, if once this ataxia appeared, no animal could survive. Few animals (1 to 2 out of 10) died without this ataxia; in these animals a reduction in locomotion and a tremor were seen before death.

It seemed that the most of males which died were affected by the ataxia within several hours following the administration of cAMP, and the recovery from the initial depression was less complete than in the females, probably because the LD 50 was higher.

There was a tendency that young (1-2 months old) females were affected by ataxia earlier (10-20 hr) and at lower doses, than older (3-4 months old) females (30-40 hr).

cAMP from different sources were tested and no difference in toxicity was observed. Sterilization of cAMP solution with Millipore filter (HA) unaffected the toxicity.

Toxicity of 5'-AMP (660 mg/kg of disodium salt, i. p., 10 females) and adenosine (500 and 1,000 mg/kg, i. p., 10 females for each) was examined, and no ataxia such as described above was observed.

Neostigmine was tested whether it could relieve the ataxia due to cAMP intoxication. Subtoxic to supratoxic doses of neostigmine bromide (0.1-1.0 mg/kg, i. p.) could not relieve the ataxia when given to the ataxic animals and rather accelerated the death of the animals.

cAMP itself affected the toxicity of cAMP, i. e., a tolerance to the toxicity of cAMP was seen. Female mice which survived after a dose of 500 mg/kg of Na-cAMP were

treated every 2 to 3 days with increasing doses of Na-cAMP, 660, 800 and 1,000 mg/kg, i. p. Most of these animals survived the dose of 1,000 mg/kg of Na-cAMP. This tolerance was lost within 3 weeks.

Twitch tension of the mouse hind limb. In the control, only slight decreases in the responses were observed within 3 hr after the start of stimulations (Fig. 1A). The mice which had been injected Na-cAMP, 660 mg/kg, i. p., were anesthetized when the ataxia began to develop. Fig. 1 B shows the time course of the decrease in tension developments during the 3 hr before the death. The response to indirect stimulation was significantly weaker than the control 3 hr before the death, while that to direct stimulation was not affected at all. Thirty minutes before the death, the indirect stimulation was no longer effective, but the direct one remained steadily effective.

Conduction ability of the sciatic nerve. Normal action potentials could be obtained from the nerve isolated from the mice which became severely ataxic from the cAMP intoxication (660 mg/kg of Na-cAMP, i. p.). Neither the maximum amplitude nor the conduction velocity of these action potentials was different from those obtained from the nerve of normal animals, as shown in Table 2.

Electrophysiological parameters of neuromuscular transmission. As Table 3 shows, in the sciatic nerve-gastrocnemius preparations isolated from normal the mice contractile force produced by indirect stimulation declined in half an hour, but that produced by direct stimulation remained fairly constant.

The preparations obtained from cAMP-intoxicated mice developed no detectable force responding to indirect stimulation, while direct stimulation produced a distinct force, though significantly weaker than that produced in normal preparations. These results roughly

Fig. 1A. Twitch tension of the mouse hind limb elicited by direct and indirect stimulation in situ (control).
●: direct, ○: indirect. 10 animals were used. Vertical bars represent the s.e.m.

Fig. 1B. Twitch tension of the mouse hind limb elicited by direct and indirect stimulation in situ (660 mg/kg of Na-cAMP, i. p.).
 ●: direct, ○: indirect.
 Numbers in parentheses indicate the number of animals used.
 Vertical bars represent the s.e.m. Dotted lines indicate the control tension.

Table. 2 Conduction characteristics of the sciatic nerve isolated from cAMP-intoxicated, ataxic mice.
 (660 mg/kg of Na-cAMP, i. p.)

	Control	Ataxic mice
Max. amplitude of action potential (mV)	16.6 ± 3.6 (4)	16.6 ± 4.1 (4)
Conduction velocity (m/sec)	19.7 ± 0.8 (4)	20.6 ± 2.7 (4)

Table. 3 Influence of cAMP-intoxication on the contractile force produced by direct and indirect stimulation in mouse sciatic nerve-gastrocnemius preparation in vitro. (660 mg/kg of Na-AMP, i. p.)

Treatment	Stimulation	Contractile force (g)	
		10 min ^{a)}	30 min ^{a)}
Control	indirect	1.17 ± 0.13	0.21 ± 0.15
	direct	1.08 ± 0.13 (6)	0.92 ± 0.19 (3)
cAMP-intoxicated ataxic mice	indirect	<0.02	<0.02
	direct	0.48 ± 0.09* (8)	0.32 ± 0.08* (6)

Values shown are means ± s.e. m.

Figures in parentheses indicate number of preparations.

a) Time after the setting of the preparation in the organ-bath.

* Significantly different from the control ($p < 0.05$).

Acute toxicity of cyclic AMP

Table. 4 Influence of cAMP-intoxication on the electrophysiological parameters of neuromuscular transmission in mouse nerve-muscle preparations in vitro. (660 mg/kg of Na-cAMP, i. p.)

Preparation	Treatment	m. e. p. p.		e. p. p.			n
		Amplitude (mV)	Frequency (Hz)	% ^{a)}	Amplitude (mV)	Quantum content	
Sciatic nerve-gastrocnemius	Control	0.31±0.03	20.8±3.3	100	35.5±10.1	108±28	8–11
	cAMP	0.29±0.04	37.8±5.9*	33	14.5±3.3	44±8*	12–19
Phrenic nerve-diaphragm	Control	0.46±0.08	10.3±2.1	100	39.1±4.8	113±18	8–11
	cAMP	0.33±0.05	11.5±3.2	50	15.2±3.3*	72±20	10–15

Values shown are mean±s.e.m. n: Number of preparations and of observations.

a) (Number of preparations from which e.p.p. was recorded / number of preparations used) × 100.

*: Significantly different from the control (p<0.05).

coincide with those of the in situ studies.

Results of the studies on the electrophysiological parameters of neuromuscular transmission are summarized in Table 4. Amplitudes of m.e.p.p. recorded from the preparations of cAMP-intoxicated mice did not differ from those of normal mice. No difference was found in the amplitudes between the m.e.p.p.'s recorded from the preparations isolated from normal and cAMP-intoxicated animals, although the frequency was higher in the gastrocnemius preparation isolated from cAMP-intoxicated animals than those isolated from normal animals. On the other hand, e.p.p. could not be recorded from many of the preparations isolated from cAMP-intoxicated animals, although it was recorded without any exception from the preparations isolated from normal animals. The mean amplitude of the e.p.p. recorded from the preparations isolated from cAMP-intoxicated animals was significantly smaller than that in the preparations isolated from normal animals. Accordingly the approximate quantum content calculated from mean m.e.p.p. and e.p.p. recorded was significantly smaller in the preparations isolated from cAMP-intoxicated animals. The resting membrane potential was little affected by the intoxication.

Acetylcholine determination. The muscles of the animals pretreated with cAMP (660 mg/kg of Na salt, i.p.) were isolated when the ataxia substantially progressed. The ACh content of the muscles isolated from ataxic animals (14.1±1.7 n mole/g wet weight, 30 animals) did not differ from that isolated from normal animals (14.1±1.5, 25 animals).

DISCUSSION

Henion and Sutherland (1967) noted using a small number of mice depressive symptoms following high doses of cAMP and a partial recovery from it, as confirmed in the present studies. They found the animals dead next morning, and had no chance to observe lethal symptoms.

The delayed ataxia found in the present studies was almost inevitably followed by death, and seemed to closely relate to the cause of death. It is suggested that the toxicity

of cAMP may not be ascribed to that of metabolites of cAMP, since adenosine and 5'-AMP at doses comparable to the toxic dose of cAMP did not cause the ataxia nor death.

The ataxia caused by cAMP may be due mainly to the dysfunction of neuromuscular transmission, as suggested by the fact that the tension development in the hind limb produced by sciatic nerve stimulation *in situ* was specifically affected as compared with that produced by direct stimulation of hind limb muscles. Since the amplitude of the m.e.p.p. was not decreased, the sensitivity of the end-plate to ACh is likely to be unaffected. The conduction ability of the sciatic nerve trunk and the ACh content in the muscles were also unaffected. It is suggested, therefore, that the main cause of transmission disturbance might possibly be a decrease in transmitter release following nerve impulse or a failure of conduction near the nerve terminals. Although the possibility of contribution of disturbance of central function could not be ruled out, the dysfunction of neuromuscular transmission found in the present studies may sufficiently explain the ataxia. The fact that neostigmine could not relieve the ataxia seems to be contradictory to these explanation, and remained to be further elucidated.

Although an involvement of cAMP in neuromuscular transmission has been proposed (Breckenridge et al., 1967; Goldberg and Singer, 1969; Wilson, 1974), the dysfunction of neuromuscular transmission found in the present investigations seems unlikely to relate to the proposed role of cAMP, if the delayed appearance of ataxia is taken into account. Some secondary effects due to damage of some other organ or tissue than neuromuscular junction may have to be considered as the cause of neuromuscular dysfunction rather than primary toxic effects of cAMP on the neuromuscular junction. Studies on this possibility are in progress.

ACKNOWLEDGEMENTS. The authors wish to express their thanks to Dr. Hiroshi Saito, Department of Chemical Pharmacology, for his excellent advice on technical aspects of the ACh determination.

REFERENCES

- Breckenridge, B. McL., Burn, J.H., and Matschinsky, F.M. (1967): Theophylline, epinephrine and neostigmine facilitation of neuromuscular transmission. *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 1893-1897.
- Goldberg, A.L., and Singer, J.J. (1969): Evidence for a role of cyclic AMP in neuromuscular transmission. *Ibid.* **64**, 134-141.
- Henion, W.F., and Sutherland, E.W. (1967): Effects of derivatives of adenosine 3', 5'-phosphate on liver slices and intact animals. *Biochim. Biophys. Acta*, **148**, 106-113.
- Litchfield, J.R., and Wilcoxon, F. (1949): A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exptl. Therap.*, **96**, 99-113.
- Maruyama, Y., and Hosoya, E. (1975): Determination of endogenous acetylcholine by pyrolysis gas-chromatography and by gas-mass-spectrometry and its application. *Igaku-no-Ayumi*, **92**, 43-52 (in Japanese).
- Wilson, D.F. (1974): The effects of dibutyryl adenosine cyclic 3', 5'-monophosphate, theophylline and aminophylline on neuromuscular transmission in the rat. *J. Pharmacol. Exptl. Therap.*, **188**, 447-452.