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J. Pharm. Pharmacol. 1986, 38: 153–155
Communicated June 18, 1985

A comparison of the antagonisms by neostigmine and diaminopyridine against the neuromuscular block caused by cobrotoxin and (+)-tubocurarine

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Cobrotoxin was about 11-fold more potent than (+)-tubocurarine on a weight basis in blocking neuromuscular transmission in mouse isolated phrenic nerve-diaphragm preparations. Neostigmine and diaminopyridine increased the concentrations of cobrotoxin for 70% inhibition of indirect contraction by 290 and 320%, and increased those of (+)-tubocurarine by 180 and 230%, respectively. More than additive increases were obtained when neostigmine and diaminopyridine were used simultaneously. Cobrotoxin, however, was only 6-fold more toxic than (+)-tubocurarine after intraperitoneal injection in mice. The lethal dose of (+)-tubocurarine was increased by 80% when both antidotes were used together, but only by 15–20% when used alone. In contrast, the lethality of cobrotoxin was not decreased by these drugs. Unexpectedly, the time to death after treatment with cobrotoxin was shortened when mice were pretreated with these antidotes.

One of the major toxic effects upon envenomation with cobra venom is the respiratory paralysis due to neuromuscular block (see Campbell 1979; Chang 1979). Most cobra venoms contain basic polypeptide neurotoxins (see Karlsson 1979) which block neuromuscular transmission by acting, like (+)-tubocurarine, on the postsynaptic acetylcholine receptor of the motor endplate (see Chang 1979). Neostigmine, a typical anticholinesterase drug used in anaesthesiology to antagonize (+)-tubocurarine-paralysis, was ineffective in overcoming the effects of cobra venom in the completely paralysed dog although there was distinct and immediate improvement if it was administered during the stage of recovery (Gode et al 1968). Similar observations were made in human envenomation (Kumar & Usgaonkar 1968; Banerjee et al 1972). Aminopyridines, which increase the release of acetylcholine by an inhibition of the K^+-conductance, have been introduced as antidotes against (+)-tubocurarine (Bowman 1982). We have compared the antidotal efficacies of neostigmine and 3,4-diaminopyridine against crude cobra (*Naja naja atra*) venom and its purified neurotoxin (cobrotoxin) with those against (+)-tubocurarine in isolated phrenic nerve-diaphragm preparations and lethality in mice. The results have revealed that both antidotes were effective equally against the neuromuscular block caused by cobrotoxin or (+)-tubocurarine in isolated preparations. However, no antidotal effect to cobrotoxin could be observed in-vivo.

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Materials and methods

Nerve-muscle preparations in-vitro. The phrenic nerve-diaphragm preparation, isolated from 20–25 g NIH or ICR mice of either sex, was incubated in 10 ml Tyrode solution (mM: NaCl 137, KCl 2·7, $CaCl_2$ 1·8, $MgCl_2$ 1·1, $NaHCO_3$ 11·9, $NaHPO_4$ 0·33, glucose 11·2) at 37 °C and oxygenated with 95% O_2 and 5% CO_2. Contractile responses were evoked by indirect stimulation of the nerve with supramaximal single pulses (pulse duration ≤0·1 ms) at 0·1 Hz and recorded isometrically by means of strain gauge force-displacement transducers (FT ·03).

Assay of antidote efficacy in-vitro. The isolated diaphragms were treated with appropriate concentrations of either cobrotoxin (0·012 μM) or (+)-tubocurarine (1·2 μM) to produce 70% depression of the contractile response at steady state. Then neostigmine (0·3–1·7 μM) or 3,4-diaminopyridine (4·5–5 μM) was added to restore the twitch contraction. The concentration of cobrotoxin or (+)-tubocurarine was then increased until the twitch contraction was depressed again to the same level. The antidote efficacy is defined as the ratio of the concentration of (+)-tubocurarine (or cobrotoxin) required to produce the same extent of block in the presence over that in the absence of antidotes.

Lethal toxicity in mice. Mice of either sex (NIH strain), 15–20 g were used. Groups of mice were injected (i.p.) with cobrotoxin or (+)-tubocurarine with or without pretreatment (i.p.) with neostigmine methylsulphate (0·25 μg g^{-1}) plus atropine sulphate (10 μg g^{-1}) and/or 3,4-diaminopyridine (1 μg g^{-1}). The LD50 was calcu-

lated from the mortality within 12 h after toxin injection according to Litchfield & Wilcoxon (1949).

Toxin and drugs. Cobrotoxin was isolated and purified from the venom of *Naja naja atra* (Taiwan cobra) collected in this laboratory according to the method of Lo et al (1966). Neostigmine methylsulphate (vagostigmine) was purchased from Shionogi Pharmac. Co. (Japan), (+)-tubocurarine from Asta-Werke AG (FRG) and diaminopyridine from Koch-Light Lab. (Colnbrook, England).

Data are shown as mean ± s.e. unless otherwise indicated.

Results

Neuromuscular block by cobrotoxin. Cobrotoxin produced a slowly progressive neuromuscular block of the mouse isolated diaphragm preparation. The effect was slowly reversible on washout of the toxin. Complete block was produced by cobrotoxin at about 0·014 μM. Both neostigmine and diaminopyridine were fully effective in restoring the neuromuscular transmission to control level. Cobrotoxin was about 100 times more potent than (+)-tubocurarine on a molar basis in producing neuromuscular block of the mouse diaphragm preparations (Table 1).

Table 1. Antidote efficacies of neostigmine and diaminopyridine against cobrotoxin and (+)-tubocurarine in-vitro.

Toxin	Antidote	Toxin concn for 70% N–M block: Without antidote (A) (μM)	With antidote (B) (μM)	Antidote efficacy (B/A)
Cobrotoxin	Neostigmine[a]	0·011±0·001	0·042±0·006*	3·89±0·18
	Diaminopyridine[b]	0·012±0·001	0·054±0·007*	4·23±0·36
	Neostigmine + diaminopyridine	0·012±0·001	0·210±0·013*	18·70±0·85
(+)-Tubocurarine	Neostigmine	1·190±0·23	3·320±0·59*	2·80±0·05
	Diaminopyridine	1·050±0·10	3·490±0·24*	3·34±0·11
	Neostigmine + diaminopyridine	1·120±0·17	11·400±1·50*	10·20±0·85

[a] 0·3–1·7 μM. [b] 4·5–5 μM.
*$P < 0.05$ vs without antidote.

Antidotal efficacy of neostigmine and diaminopyridine in-vitro. The antidote efficacy of neostigmine (0·3–1·7 μM) against cobrotoxin was 3·9 ± 0·2 whereas that against (+)-tubocurarine was 2·8 ± 0·1. The concentration of neostigmine used (0·3–1·7 μM) produced about 80–90% inhibition of acetylcholinesterase activity in the homogenate of mouse diaphragms (Chang & Su 1982) and the antidote efficacy could not be further increased by increasing the concentration.

Diaminopyridine (4·5–5 μM) was slightly more effective than neostigmine in antagonizing the neuromuscular blocking effects of both cobrotoxin and (+)-tubocurarine. Again, the antidote efficacy to cobrotoxin (4·2 ± 0·4) was higher than that to (+)-tubocurarine (3·3 ± 0·1). When both neostigmine and diaminopyridine were added simultaneously, the antidote efficacy was significantly higher than expected from an additive antidote efficacy for cobrotoxin (Table 1) as previously reported for (+)-tubocurarine (Chang et al 1984). Higher concentrations of diaminopyridine were not used because the contractility of muscle itself was also increased and would result in an exaggerated antidote efficacy.

Antagonism against lethal toxicity. Mice injected (i.p.) with (+)-tubocurarine (0·5–0·7 μg g^{-1}) died within 19 ± 5 min of respiratory failure. On the other hand, the mice died at 64 ± 9 min (n = 5) after i.p. injection of 0·12 μg g^{-1} cobrotoxin. The approximate median lethal doses for cobrotoxin and (+)-tubocurarine were 0·10 and 0·6 μg g^{-1}, respectively. When mice were pretreated (i.p.) with 0·2 μg g^{-1} neostigmine plus 10 μg g^{-1} atropine or with 1 μg g^{-1} diaminopyridine 10 min before the challenge of (+)-tubocurarine, the median lethal doses were increased by about 15–20% (Table 2). When both neostigmine and diaminopyridine were administered simultaneously at one half of each their individual doses, the median lethal dose was raised approximately to 1·1 μg g^{-1} from 0·6 μg g^{-1}. In contrast, the lethality of cobrotoxin was not appreciably changed by pretreatment with neostigmine or diaminopyridine, either used alone or in combination (Table 2). Unexpectedly, the time to death was shortened from 64 ± 9 min to 42 ± 3 (n = 8), 44 ± 6 (n = 7) and 33 ± 3 min (n = 7) for the mice pretreated with neostigmine, diaminopyridine alone and in combination, respectively. The doses of neostigmine and diaminopyridine used for the simultaneous application were the maximum tolerable. Trials to overcome the lethality of cobrotoxin by supplementing neostigmine and/or diaminopyridine at 30 min

Table 2. Effect of neostigmine and diaminopyridine on the lethality of (+)-tubocurarine and cobrotoxin in mice.

Toxin	Dose (i.p. μg g^{-1})	Lethality (died/tested): Control	Neostigmine[a]	Diaminopyridine[a]	Neostigmine + diaminopyridine[a]
(+)-Tubocurarine	0·5	1/8	1/8	0/8	—
	0·6	7/12	1/8	1/10	—
	0·7	8/8	17/38	6/12	1/4
	1·0	—	12/12	8/8	2/8
	1·2	—	—	—	6/8
	1·5	—	—	—	4/4
Cobrotoxin	0·08	0/6	—	—	—
	0·1	4/8	—	—	—
	0·12	9/10	8/8	7/7	7/8

[a] Pretreated (i.p.) 10 min before the challenge of (+)-tubocurarine or cobrotoxin, with 0·2 μg g^{-1} neostigmine methyl sulphate, 1 μg g^{-1} diaminopyridine or with 0·1 μg g^{-1} neostigmine plus 0·5 μg g^{-1} diaminopyridine. Atropine sulphate (10 μg g^{-1}) was also given when neostigmine was administered.

intervals were also unsuccessful. Neostigmine and diaminopyridine were also ineffective in antagonizing the lethal toxicities of crude cobra venom and α-bungarotoxin (unpublished observations).

Discussion

In the mouse isolated phrenic nerve-diaphragm, cobrotoxin is about one hundred times more potent than (+)-tubocurarine on a molar basis and eleven times more potent on a weight basis. Nevertheless, the cobrotoxin-induced neuromuscular block was antagonized by neostigmine and diaminopyridine equally well as the (+)-tubocurarine-induced block. These results are in accordance with the view that cobrotoxin blocks neuromuscular transmission by the same mechanism as (+)-tubocurarine, i.e. by occupation of the postsynaptic acetylcholine receptor (Chang 1979). The slightly better antagonism against cobrotoxin might be due to the prolonged time of experiments (4–7 h) due to the slow equilibration with cobrotoxin. A spontaneous recovery from the neuromuscular block by cobrotoxin was found to occur 6–7 h after the isolation of the nerve-muscle preparation (unpublished observation). The synergism between neostigmine and diaminopyridine is understandable since they act by different mechanisms.

In contrast to the in-vitro experiments, both neostigmine and diaminopyridine showed rather limited antidote efficacy in-vivo against (+)-tubocurarine and were almost ineffective against cobrotoxin. This may be attributed partly to the higher rate of activity of the phrenic nerve against cobrotoxin in-vivo. With high frequency stimulation, the antagonistic effects of neostigmine and aminopyridines were reduced significantly (Chang et al 1984). The ineffectiveness of neostigmine and diaminopyridine against cobrotoxin in-vivo, could be due to a change of the absorption or other pharmacokinetic property of cobrotoxin induced by these antidotes, causing a reduction of time to death.

In conclusion, our results indicate that neostigmine and diaminopyridine, either used alone or in combination, were not effective as antidotes against envenomation with cobra venoms in spite of effectiveness in-vitro.

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