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NEUROMUSCULAR EFFECTS OF FOUR PHOSPHOLIPASES A_2 FROM THE VENOM OF *PSEUDECHIS AUSTRALIS*, THE AUSTRALIAN KING BROWN SNAKE

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S. L. GEH, E. G. ROWAN and A. L. HARVEY. Neuromuscular effects of four phospholipases A_2 from the venom of *Pseudechis australis*, the Australian king brown snake. *Toxicon* **30**, 1051–1057, 1992.—Four homologous single chain phospholipases A_2 (Pa-1G, Pa-5, Pa-12C and Pa-15) were tested for neuromuscular effects on chick biventer cervicis and mouse hemidiaphragm nerve–muscle preparations. The four isozymes blocked directly elicited (mouse hemidiaphragm) and indirectly elicited (mouse and chick nerve–muscle preparations) twitch responses in concentrations of 1–30 μg/ml. The order of potency seen in both types of preparations was Pa-1G = Pa-5 > Pa-12C > > Pa-15. All four isozymes caused slow-onset, sustained contractures and reduction of muscle membrane potentials. In the chick preparation, responses to acetycholine, carbachol and KCl were reduced by exposure to the toxins. It is concluded that the toxins act primarily postsynaptically to depress muscle contractility, perhaps by directly damaging muscle fibres. The order of potency agrees with their phospholipase A_2 activity. Pa-1G is unusual because it is an acidic molecule, most toxic phospholipases being basic.

INTRODUCTION

Pseudechis australis (king brown snake) is the largest venomous snake in Australia. Its venom contains postsynaptic curaremimetic neurotoxins (TAKASAKI and TAMIYA, 1985) and several phospholipases A_2 (TAKASAKI and TAMIYA, 1982). Thirteen isoenzymes of phospholipase A_2 have been isolated and characterised (NISHIDA *et al.*, 1985*a*, TAKASAKI *et al.*, 1990*a*) and eight have been sequenced (NISHIDA *et al.*, 1985*b*; TAKASAKI *et al.*, 1990*a*). Although the sequences are highly homologous, there are marked differences in the phospholipase A_2 activity and lethality of the isoforms: enzyme activity varies from 75 to 10.500 units mg, and LD_{50} varies from 0.09 to 6.8 μg/g body weight (TAKASAKI *et al.*, 1090*a*). Hence, these isoenzymes from *Pseudechis australis* provide a useful set of molecules with which structure–activity relationships of toxic phospholipases A_2 can be investigated.

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As other toxic phospholipases A_2 from snake venoms can have a variety of pharmacological actions, including direct myotoxicity and presynaptic blockade of acetycholine release (for a review see HARRIS, 1991), it is necessary to go beyond correlations of enzymatic properties and lethality when examining structure–activity relationships. Characterisation of neuromuscular effects is thus essential. Previously, we have examined the actions of three phospholipases A_2 (Pa-10A, Pa-11 and Pa-13) from *Pseudechis australis* on isolated nerve–muscle preparations (ROWAN *et al.*, 1989). We have continued this study with Pa-1G, Pa-5, Pa-12C and Pa-15. Some of these results were reported in a preliminary form at the 5th South East Asia/Western Pacific Regional Meeting of Pharmacologists, Hong Kong, July 1991 (GEH *et al.*, 1991*a*) and at the 10th World Congress on Animal, Plant and Microbial Toxins, Singapore, November 1991 (GEH *et al.*, 1991*b*).

MATERIALS AND METHODS

Toxins

Pa-1G, Pa-5, Pa-12C and Pa-15 were gifts from Drs N. Tamiya and C. Takasaki (Department of Chemistry, Tohoku University, Sendai, Japan). They had been isolated as described by TAKASAKI *et al.* (1990*a*), and behaved as homogeneous proteins on high pressure liquid chromatography.

Chick biventer cervicis nerve–muscle preparations

Biventer cervicis muscles and associated nerves were removed from 4–10 day-old chicks, and mounted in 10 ml tissue baths with a resting tension of approximately 1 g in a physiological salt solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 11.1. The solution was maintained at 32°C and pH 7.3, and bubbled with oxygen containing 5% CO_2. Twitches were evoked by stimulating the motor nerves at the desired frequency with pulses of 0.2 msec duration and a voltage greater than that which produced maximal twitch. Contractions to exogenously applied acetylcholine ($1–3 \times 10^{-3}$ M), carbachol ($2–4 \times 10^{-5}$ M) and KCl ($1–3 \times 10^{-2}$ M) were obtained prior to the addition of the toxin and at the end of the experiment. In some experiments, measurements of muscle fibre membrane potentials were made after exposure to toxins. Time-matched control preparations were also tested. Membrane potentials from several fibres in each muscle tested were recorded using glass microelectrodes (filled with 3 M KCl, resistance 5–15 MΩ) placed inside the muscle fibre. The potential difference between the silver silver chloride reference electrode in the bath and the recording electrode was measured by a high impedance unity gain electrometer (W-P instruments, model 750) and displayed on a dual beam storage oscilloscope.

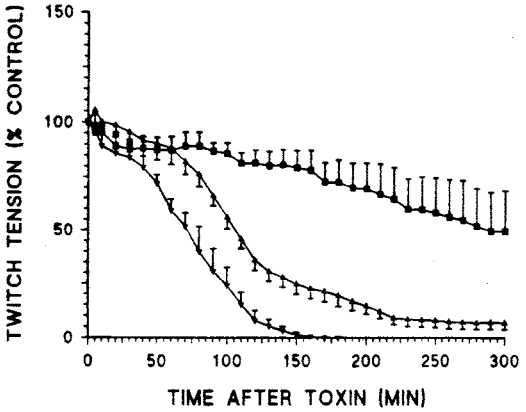

FIG. 1. BLOCKADE OF TWITCHES OF CHICK BIVENTER CERVICIS MUSCLE PREPARATIONS BY Pa-12C. ■, 3 μg/ml; ▲, 10 μg/ml; ▼, 30 μg/ml. Preparations were stimulated indirectly at 0.1 Hz. Symbols represent means of experiments on four to six preparations; vertical bars indicate S.E.

Mouse phrenic nerve–hemidiaphragm preparations

Hemidiaphragms and attached phrenic nerves were removed from male mice (20–25 g, Balb C strain). The preparations were mounted in 10 ml organ baths and attached to stimulating electrodes that enabled the preparations to be stimulated directly or indirectly. The composition of the physiological solution was the same as used for the biventer cervicis preparations, and experiments were performed at 32°C. For indirect stimulation, the phrenic nerve was stimulated with pulses of 0.2 msec of sufficient strength to produce a maximal twitch. For direct muscle stimulation, pulses of 2 msec were applied at 0.1 Hz with sufficient strength to produce a maximal twitch. Some preparations were stimulated alternately, indirectly and directly, at a frequency of 0.05 Hz. Prior to the addition of the test compounds, tubocurarine was always added to ensure that activation of nerve endings by the direct stimulation did not contribute to the overall tension recorded in response to the direct stimulation; thereafter, the tubocurarine was washed out for approximately 30 min. In some experiments, membrane potentials were measured after exposure to toxins, as described above for chick preparations.

Statistics

Values for 50% blocking times and responses to agonists are given as means ± S.E. of groups of at least four experiments. Differences between means were tested using Student's *t*-test.

RESULTS

Chick biventer cervicis preparations

Pa-1G, Pa-5 and Pa-12C reduced responses to indirect stimulation in a time- and concentration-dependent manner. Figure 1 shows the development of block with three concentrations of Pa-12C. The effects of Pa-1G and Pa-5 were similar, although they are about three to five times more potent at blocking twitch responses than was Pa-12C (Fig. 2). Pa-15 could also block twitch responses to nerve stimulation, but it was much less active than the other three isoenzymes: 30 μg/ml was required to cause 50% block in 5 hr,

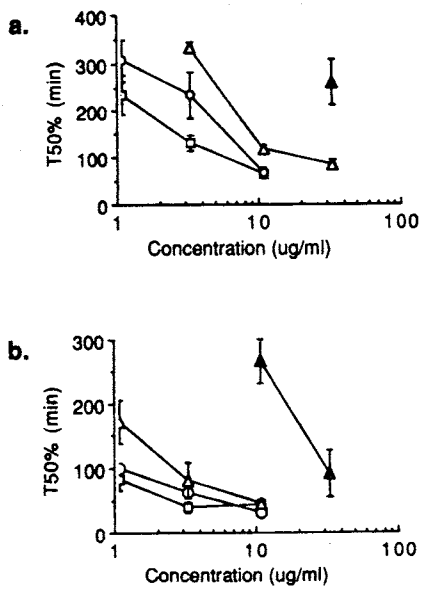

FIG. 2. EFFECTS OF FOUR PHOSPHOLIPASES ON TWITCH RESPONSES OF CHICK BIVENTER AND MOUSE HEMIDIAPHRAGM PREPARATIONS.

(a) Time to 50% block of twitches to indirect stimulation of chick biventer cervicis preparations; (b) time to 50% block of twitches to indirect stimulation of mouse hemidiaphragm preparations. □, Pa-1G; ○, Pa-5; △, Pa-12C; ▲, Pa-15. Symbols represent means of four to six experiments; vertical bars indicate S.E.

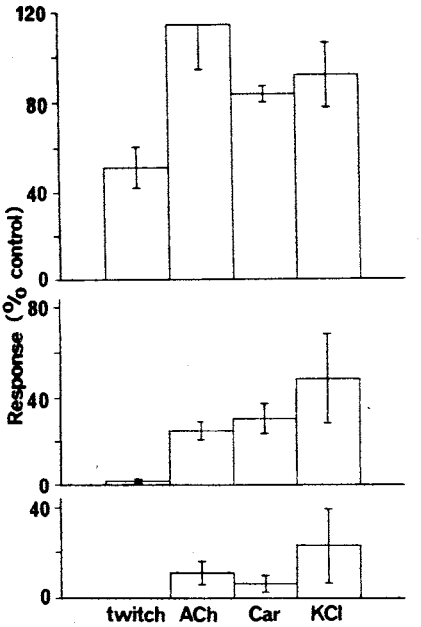

FIG. 3. EFFECTS OF Pa-1G ON RESPONSES OF CHICK BIVENTER CERVICIS PREPARATIONS TO INDIRECT STIMULATION (TWITCH), ACETYLCHOLINE (ACh), CARBACHOL (Car), AND KCl.
Upper, 1 μg/ml; middle, 3 μg/ml; lower, 10 μg/ml. Columns represent means of four to six experiments; vertical bars indicate S.E.

and 10 μg/ml caused less than 20% block in 5 hr. The relative blocking potency of the four toxins was compared by measuring time to 50% block of twitch responses (Fig. 2a).

Responses to acetylcholine, carbachol and KCl were tested after complete block of twitch or after 5 hr, even when the block was less than 50%. All four toxins reduced responses to the three agonists in a concentration-dependent manner, as illustrated by the effects of Pa-1G (Fig. 3).

Many preparations slowly developed a contracture following administration of the toxin. The contracture slowly waned in 20–40 min. At the end of some experiments, preparations were examined under a light microscope and resting membrane potentials were measured. There were signs of damage to the muscle fibres (supercontracted fibres, and areas with gaps between fibres and darkened dead cells), especially after exposure to the highest concentrations of the toxins. Membrane potentials in these preparations were lower than in time-matched control preparations. For example, after exposure to Pa-1G (10 μg/ml) for 5 hr, membrane potential was -10.8 ± 0.7 mV (mean $\pm$ S.D. of two preparations, 50 fibres measured in each preparation); membrane potential in a time-matched control preparation was -37.2 ± 3.0 mV (30 fibres).

Mouse hemidiaphragm preparations

All four toxins blocked twitch responses to both direct and indirect stimulation. There was normally an initial increase in twitch height of about 10–25%, lasting for 10–15 min. Thereafter, there was a progressive decrease in twitch height (Fig. 4 shows results with Pa-5). There was little difference in the inhibition of the direct and indirect twitch

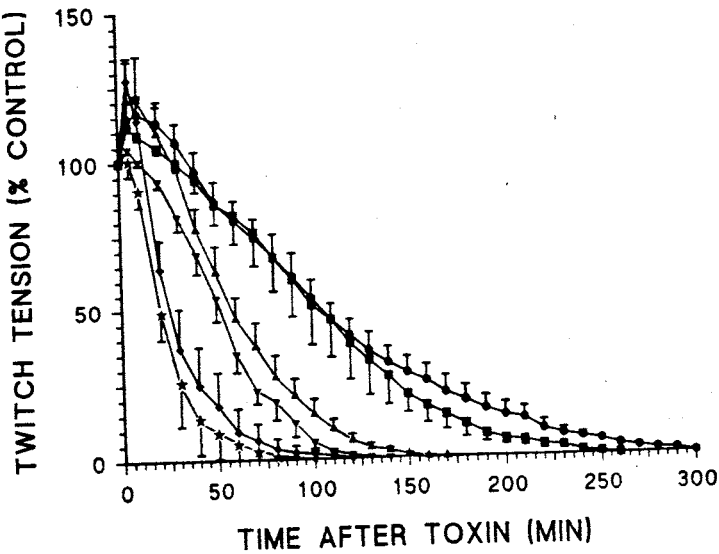

FIG. 4. EFFECTS OF Pa-5 ON TWITCH RESPONSES OF MOUSE HEMIDIAPHRAGM PREPARATIONS.
Preparations were stimulated alternately directly and indirectly at 0.05 Hz. ●, 1 μg/ml on direct stimulation; ■, 1 μg/ml on indirect stimulation; ▲, 3 μg/ml on direct stimulation; ▼, 3 μg/ml on indirect stimulation; ◆, 10 μg/ml on direct stimulation; ★, 10 μg/ml on indirect stimulation. Symbols represent means from four to six experiments; vertical bars indicate S.E.

responses. The order of blocking potency was similar to that found in the chick biventer cervicis preparation (Fig. 2b).

At the end of the twitch tension experiments, muscles were examined by light microscopy and membrane potentials were measured. The appearance of the muscles varied considerably, with some looking normal and others having areas of grossly disrupted fibres. However, membrane potentials were considerably lower in preparations that had been exposed to the toxins than in time-matched control preparations (Fig. 5). As each preparation was sampled after reaching the same level of block (just over 50%), there was

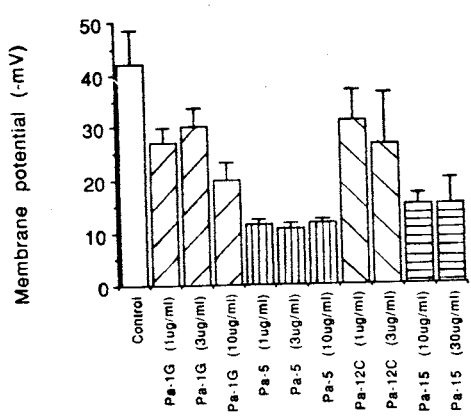

FIG. 5. EFFECTS OF THE FOUR PHOSPHOLIPASES ON MEMBRANE POTENTIALS OF MOUSE HEMIDIAPHRAGM PREPARATIONS.
Columns represent means of measurements made in two to five preparations (20–30 cells were recorded in each preparation); bars indicate S.E.

no apparent relationship between reduction in membrane potential and concentration of toxin. However, there were differences between the effects of individual toxins on membrane potential. For example, exposure for 3.5–4 hr to Pa-1G (3 μg/ml) resulted in membrane potentials of -30.1 ± 3.7 mV (mean $\pm$ S.E. of the average values from three preparations, 20–25 fibres sampled in each muscle), whereas 4 hr exposure to Pa-5 (3 μg/ml) gave membrane potentials of -10.8 ± 1.1 mV.

DISCUSSION

The four phospholipases A_2 (Pa-1, Pa-5, Pa-12C and Pa-15) from *Pseudechis australis* impaired neuromuscular function *in vitro*. There was little difference in the blocking potencies on direct and indirect stimulation of mouse diaphragm preparations; responses of chick biventer cervicis preparations to acetylcholine, carbachol and KCl were similarly affected; and the toxins caused gross muscle damage and depolarization on prolonged exposure. These results are consistent with the toxins having a predominant action on muscle fibres, rather than on neuromuscular transmission. In contrast, phospholipases A_2 Pa-10A and Pa-11 from the same venom had been previously found to have a presynaptic blocking action on acetylcholine release in addition to direct myotoxic effects (ROWAN *et al.*, 1989).

Generally, basic phospholipases A_2 are more toxic than neutral or acidic homologues (HARRIS, 1991). Pa-1G is acidic (p*I* of 6.4), but it was almost the most active of the four isoenzymes at blocking twitch responses. It is also lethal, with an LD_{50} in mice of 0.13 μg/g body weight (TAKASAKI *et al.*, 1992*a*).

The potency of the four phospholipases A_2 in producing twitch blockade in the chick and mouse preparations varied by a factor of about 30 in the order Pa-1G = Pa-5 > Pa-12C > > Pa-15. This correlates with both enzymatic activity and lethality as determined by LD_{50} after 24 hr (TAKASAKI *et al.*, 1990*a*). Although lethality of all the isozymes was associated with haemoptysis, haemoglobinuria and limb paralysis (TAKASAKI *et al.*, 1990*a*), the cause of death may differ after administration of the different isozymes. The neurotoxic effects of Pa-10A and Pa-11 were mentioned earlier (see ROWAN *et al.*, 1989), and concentrations of Pa-1G and Pa-5 that were equieffective at blocking muscle twitches were significantly different in their effects on muscle membrane potentials.

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