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THE EFFECT OF PONERATOXIN ON NEUROMUSCULAR TRANSMISSION IN THE RAT DIAPHRAGM

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Abstract: The effect of the ant venom neuropeptide – poneratoxin (PoTX) – on neuromuscular transmission in rat diaphragm tissue was studied by means of intracellular recordings of spontaneous miniature endplate potentials (MEPPs) and of nerve evoked endplate potentials (EPPs). A 2 μ M concentration of PoTX caused a pronounced but transient increase in MEPPs frequency. Moreover, within the first few minutes of PoTX administration, the area, rise time and half decay time of MEPPs gradually decreased, reaching steady values after 15–20 min. The alteration of the area, rise time and half decay time of EPPs after PoTX application was similar to that observed for MEPPs. We conclude that PoTX affects neuromuscular transmission in rat tissue, and suggest that PoTX could exert both pre- and postsynaptic effects.

Key Words: Poneratoxin, Neuromuscular Transmission, Miniature Endplate Potentials, Endplate Potentials

INTRODUCTION

Poneratoxin (PoTX) is the most active neurotoxic fraction purified from the venom of the ponerine ant, *Paraponera clavata* [1]. Purification and sequencing revealed that PoTX is a peptide of 25 amino acid residues [2]. Based on the determined sequence, a synthetic form of this toxin can be obtained.

Electrophysiological experiments on insects have demonstrated that at submicromolar concentrations, PoTX blocks synaptic transmission in the central nervous system, depolarises giant interneurons and causes fibrillation of

skeletal muscles [3]. Moreover, it has been shown that PoTX prolongs the action potential in vertebrate skeletal muscles [3]. It was proposed that this effect on the action potential could be a consequence of the activation (by PoTX) of a slow class of Na channels active at very negative potentials. Further work done on the frog skeletal muscle [4] demonstrated that PoTX acts specifically on voltage dependent Na channels, interconverting these channels from a normal (fast) to a slow mode of action. Thus, it appears that in different species of animals, PoTX may exert different effects on various tissues by interfering with the excitability of nerve and muscle cells and by affecting synaptic transmission.

In this study, we investigated the effect of synthetic PoTX on neuromuscular transmissions in rat. We report that PoTX, at a concentration of 2 μ M, significantly increases the frequency and alters the kinetics of the miniature endplate potential (MEPPs) suggesting pre- and postsynaptic effects.

MATERIALS AND METHODS

The experiments were performed on diaphragm muscle tissue of Wistar male rats, weighing 150-180 g. Before dissection, the rats were killed by ether overanesthesia. The hemidiaphragm attached to 2-3 cm long sections of the phrenic nerve was bathed in a physiological solution composed of 142 mM NaCl, 5 mM KCl, 1.5 mM CaCl_2 , 1 mM MgCl_2 , 5 mM glucose, 5 mM HEPES (pH=7.2).

In experiments in which nerve evoked endplate potentials (EPPs) were recorded, in order to avoid muscle contraction, a partial presynaptic block of the neuromuscular transmission was applied. The required degree of neuromuscular block was reached by reducing the amount of CaCl_2 (0.8 mM) and by increasing the amount of MgCl_2 (up to 10 mM) in the external medium.

Both spontaneous miniature endplate potentials (MEPPs) and EPPs were recorded at room temperature (20-23°C), using a conventional intracellular technique, using glass microelectrodes filled with 3 M KCl, with a tip resistance of 5–10 M Ω . The details of the recording were described previously [5]. The recordings were stored on magnetic tape. For the subsequent off-line analysis the signals were low-pass filtered at 2-3.15 kHz (8-pole Bessel filter, Ithaco) and sampled at 10-20 kHz, using an ADDA (IEVT IFT 308) converter, on a computer hard disk. On comparison, significant differences were not found between the time courses of the signals filtered at 2-3.15 kHz and those filtered at 3.15-5 kHz; nor were any differences observed when electrodes with different tip resistances were used in the range mentioned above. The acquisition and analysis software was kindly provided by Dr. J. Dempster (Strathclyde University, Glasgow, UK). The spontaneous and evoked postsynaptic potentials were analysed as described elsewhere [6].

The ponera toxin used in the experiments was synthesised as described in [7]. The purity of the peptides was checked by HPLC. Other chemicals used were of analytical grade.

RESULTS

In seven out of eight experiments, a significant increase ($p < 0.05$, t -Student test) in MEPP frequency was observed after the addition of the toxin at the final concentration of $2 \mu\text{M}$. Under the control conditions, the average MEPP frequency was 14.8 s^{-1} . Within the first five minutes after PoTX administration, this frequency reached a maximum average value of 71.1 s^{-1} and then showed a gradual decrease, reaching the control value within 15-20 minutes (see Fig. 1). The extent of the increase in MEPP frequency was greatly variable from one experiment to another, ranging from 40% to 2000% with a mean value of $480\% \pm 85\%$. Only in one out of eight experiments did the administration of PoTX not significantly affect the frequency of the MEPPs ($p > 0.05$).

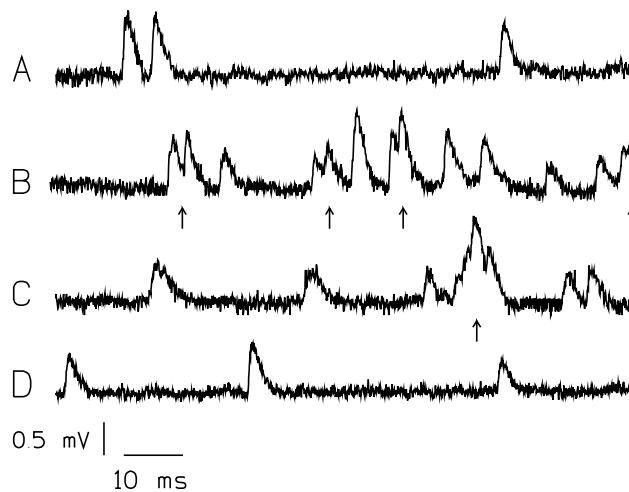


Fig. 1. MEPPs recorded in a typical experiment on the rat diaphragm: control (A) and 5 min. (B), 10 min. (C) and 30 min. (D) after application of PoTX at a final concentration of $2 \mu\text{M}$. In B and in C, "clustered" MEPPs are indicated by the arrows.

In one experiment, after the addition of PoTX, the frequency of MEPPs increased by ca. 40%, but after 25 min of treatment, the MEPPs disappeared. Ten minutes after wash-out, the MEPPs reappeared at the same frequency as observed in the control.

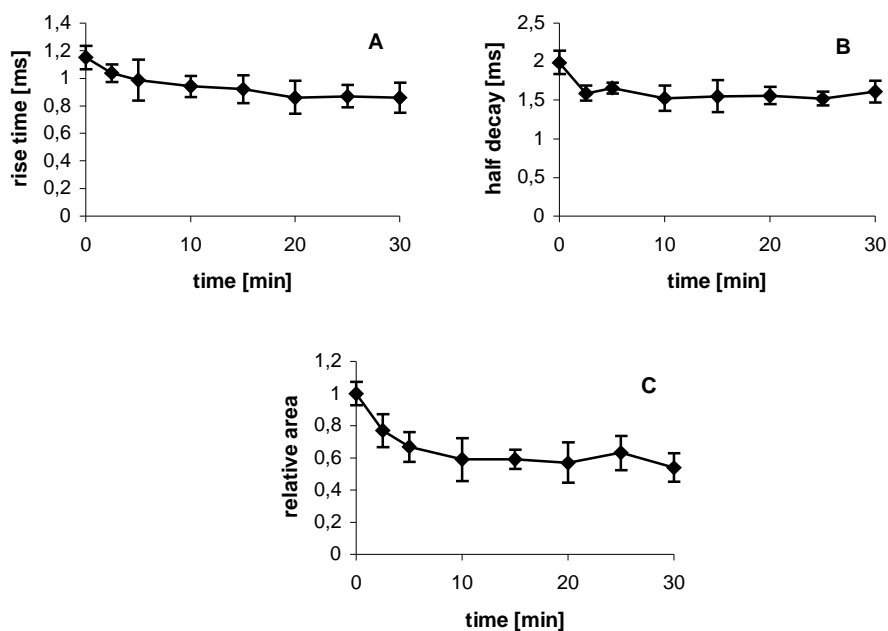


Fig. 2. Time courses of MEPP rise time (A), half decay time (B), and area (C) recorded in the rat diaphragm after the addition of 2 μ M PoTX. The zero time corresponds to the control conditions. Before averaging, the areas recorded in each experiment were normalised by dividing by the control values. Symbols and bars represent mean values \pm SD (n=8).

In all the experiments in which PoTX induced an increase in the MEPP frequency, MEPPs very often appeared in "clusters" (Fig. 1 B, C). Under the control conditions, overlapping MEPP were very rarely seen and no more than two superimposed MEPPs were observed. Since these PoTX-induced "clusters" many times consisted of as many as 3-8 MEPPs (Fig. 1 B, C), it seems unlikely that these groups could result merely from the increase in the MEPP frequency. In our control recordings, we observed a small frequency (ca. 0.5 per second) of so called giant MEPPs [8, 9, 10] but their appearance was not significantly affected by PoTX.

In all the experiments, the rising and decaying phases of MEPP were accelerated in the presence of PoTX (Fig. 2 A, B). In order to obtain more quantitative information on the effect of PoTX, the MEPP area was also measured, and as seen in Fig. 2C, the effect of PoTX on this parameter had a very similar time dependence to that observed for the rise time and half decay time of MEPP.

It must be pointed out that the resting membrane potential was not affected by the administration of PoTX (not shown); it remained stable during the experiments (no more than 3 mV depolarisation from -60 mV was seen during 30 min of recordings). Thus, the PoTX-induced changes in the MEPP kinetics (Fig. 2) are unlikely to result from a modification of the electrical driving forces for ions.

In three experiments, the nerve evoked EPP was recorded at a frequency of 0.3 Hz throughout the experiment. In this case, the concentration of magnesium ions in the bath solution was increased to avoid EPP-induced muscle contraction. In these experiments, the EPP amplitude after PoTX treatment decreased similarly to that of MEPPs (see Tab. 1). Thus, the quantal content was not significantly affected ($p > 0.5$, t -Student test). PoTX had the same effect on the time course and on the area of EPP as on that of MEPPs. The fractional decrease in EPP area was thus indistinguishable from that of MEPP area. The rising and falling phase of EPPs in the presence of PoTX was shortened to the same extent as for MEPPs (Table 1).

Tab. 1. Comparison of the relative decrease of MEPP and EPP amplitude, area, rise time, and half decay time. Each column was normalised by dividing by the appropriate control values.

Time [min]	Amplitude		Area		Rise time		Half decay	
	MEPP	EPP	MEPP	EPP	MEPP	EPP	MEPP	EPP
3	0.88	0.91	0.76	0.82	0.90	0.92	0.80	0.78
5	0.79	0.79	0.66	0.74	0.85	0.87	0.83	0.77
10	0.78	0.73	0.59	0.70	0.81	0.81	0.76	0.74
15	0.76	0.67	0.59	0.59	0.80	0.79	0.78	0.75
20	0.76	0.67	0.57	0.59	0.74	0.79	0.78	0.74
25	0.75	0.64	0.63	0.54	0.75	0.77	0.76	0.73
30	0.76	0.65	0.54	0.51	0.74	0.76	0.80	0.74

DISCUSSION

The results presented show that synthetic PoTX modifies the rat neuromuscular transmission presynaptically, by increasing the frequency of spontaneous quantal release, and postsynaptically, affecting the time course of MEPPs and EPPs. While the time course of MEPPs was significantly affected in all the experiments, the PoTX effect on MEPP frequency was variable from one preparation to the next, despite the same toxin concentration having been used. This finding suggests that the effect of PoTX, in our experimental conditions, is dependent on some factor(s) limiting the efficacy of toxin action. Duval *et al.*

[4] observed that in a preparation of frog muscles, the effect of PoTX on Na channels was much stronger when the muscle membrane was cleaned by enzymatic treatment, suggesting that the accessibility of the drug could be a limiting factor. In our experiments, we did not use enzymes to clean the preparation, and connective tissue was only mechanically removed from the surface of the muscle. It is thus possible that the residual connective tissue in the preparation could have affected the accessibility of the drug.

The PoTX-induced increase in MEPP frequency observed in the first minutes after drug administration was followed by a progressive decrease in the frequency, which, in spite of the presence of the drug in the bath, returned to the control values within 10-20 min. Only in one experiment was the increased MEPP frequency followed by a disappearance of MEPPs that was restored ca. 10 min. after drug wash-out. The EPP quantal content was not significantly affected by the toxin, suggesting that the recruitment of vesicles for exocytosis during EPP was not significantly affected. However, as explained above, in these experiments an increased concentration of magnesium had to be used to avoid EPP induced muscle contraction, and it is known that this factor itself affects the quantal release. Therefore, we cannot exclude the possibility that the increase in magnesium concentration could, at least partially, obscure the effect of PoTX. It is thus possible that in other experimental conditions different results could be observed.

In our experiments, we observed that while the effect of PoTX on the MEPP frequency was transient, the addition of this drug caused persistent changes in the area and in both the rise and half-decay times of MEPPs and EPPs. These findings suggest that PoTX exerts both pre- and postsynaptic effects. This is further confirmed by the observation that the extent of the PoTX-induced decrease of the MEPP area and of the time constants was very similar in all the performed experiments, whereas the PoTX effect on the MEPP frequency varied substantially from one experiment to another. Thus, it seems possible that the increase in the MEPP frequency reflects a presynaptic effect of PoTX and that the changes in the MEPP kinetics and area are a consequence of the postsynaptic action of the toxin. These observations suggest that the presence of connective tissue which we suppose (see above) to limit the efficacy of the toxin action is more critical at the presynaptic than at postsynaptic side.

Piek *et al.* [3] reported that PoTX, at micromolar concentrations, induced spontaneous and prolonged action potentials in frog muscle fibres and, in a later paper [4], it was shown that this phenomenon was due to PoTX-induced change in the Na channel gating mode. In our experiments, PoTX did not affect the resting membrane potential and did not induce any spontaneous action potentials which, under our experimental conditions, could be detected by some extra-occurring EPP or some muscle action potentials. The lack of spontaneous action potentials in the presence of PoTX in our experiments may suggest that the effect of PoTX on the sodium channels in rat and frog muscles is different.

The increase in the MEPP frequency caused by PoTX resembles the effect of α -latrotoxin, a spider venom known to induce massive quantal release of ACh in vertebrate cholinergic neuromuscular junctions [11]. However, unlike the effect of α -latrotoxin, PoTX only caused a transient increase in frequency. It was also found that Thr⁶-bradykinin, a component of wasp venom, was able to irreversibly block the nicotinic synaptic transmission in the insect central nervous system by means of depletion of the neuronal terminal transmitter store [12, 13] (see also [1, 14] for review). However, Thr⁶-bradykinin action appears to be different from that of PoTX observed in our experiments, since the PoTX-induced increase in MEPP frequency was transient, and the EPP quantal content was not significantly affected. Recently, it has been reported that the phosphatase blocker, okadaic acid, induced a transient increase in the MEPP frequency [15], similarly to that observed in our experiments. We therefore cannot exclude the possibility that PoTX exerts its effect on MEPP frequency indirectly, by interfering with the releasing machinery via the second messenger system.

In conclusion, these data show that PoTX affects cholinergic neuromuscular transmission but the molecular mechanism underlying the observed effect of the toxin has still to be elucidated. More studies, possibly using other experimental approaches, are needed to further understand this problem.

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