In vitro inactivation of the neurotoxic action of β-bungarotoxin by the marine natural product, manoalide[1]

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Summary. The irreversible neurotoxic action of β-bungarotoxin (β-BuTx) can be prevented by preincubation of the toxin with manoalide, a non-steroidal anti-inflammatory agent. Manoalide was also found to inactivate purified phospholipase A_2 and thus prevent hydrolysis of phosphatidylcholine. PLA_2 is a component found in several neurotoxic venoms and is also a rate limiting enzyme important in phospholipid metabolism and prostaglandin synthesis in man.

Manoalide is a non-steroidal anti-inflammatory substance isolated from the sponge, *Luffariella variabilis*. This type of sesterterpenoid represents a rare molecular structure having no known analog reported in the literature (fig. 1)[4]. In studies in mice, manoalide was found to be analgesic and to inhibit phorbol-induced inflammation but not arachidonic acid induced inflammation. These studies suggested that manoalide may act prior to the cyclo-oxygenase step in prostaglandin synthesis, possibly at the level of PLA_2[5]. We report here results of experiments indicating that manoalide acts directly by inactivating PLA_2. In one study, manoalide prevented the neurotoxic action of β-BuTX on the rat phrenic nerve-diaphragm preparation. In a biochemical study, manoalide prevented the hydrolysis of phosphatidylcholine by purified PLA_2. Manoalide has no structural similarity to any known anti-inflammatory agent and has a pharmacological spectrum of activity different from the known PLA_2 inhibitors.

Materials and methods. Manoalide was isolated, purified and the structure verified by D.J. Faulkner of the Scripps Institution of Oceanography, La Jolla, California. β-BuTX, quinacrine dihydrochloride (mepacrine), PLA_2 purified from bee venom and DL α-phosphatidylcholine dipalmitoyl were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Rat phrenic nerve-hemidiaphragm preparation. The right and left hemidiaphragm sections were dissected from male Sprague-Dawley rats (140–250 g) and set up according to the method of Bülbring[6]. Each hemisection was suspended in Krebs-Henseleit solution maintained at 32 °C and aerated with 95% O_2 and 5% CO_2. Isometric twitch responses were recorded following supramaximal stimuli to the phrenic nerve (0.2 Hz). After recording control responses, β-BuTX, or β-BuTX pre-incubated with manoalide or mepacrine, was added to the solution bathing the preparation. Propylene glycol, the vehicle for manoalide, was present in all control experiments. The average time to reach 50% paralysis (TI_{50}) was determinated for each treatment group and statistical comparisons were made on the basis of students t-test.

Measurements of purified phospholipase A_2 activity. Solutions of manoalide dissolved in propylene glycol were pre-incubated at 41 °C with phospholipase A_2 for 1 h. Control tubes contained an equivalent volume of propylene glycol. 100 µl of the PLA_2-manoalide, or PLA_2-control solution, was then added to 10 ml of substrate suspension [1.36 mM phosphatidylcholine in 1 mM $CaCl_2$ and approximately 133 µl Triton X100 (pH = 7.4, temperature 41 °C)]. The initial velocity of hydrolysis of phosphatidylcholine was measured under nitrogen with a pH stat as the amount of 0.005 N NaOH consumed in 1 min. The responses obtained are linear for several min using these concentrations of enzyme and substrate.

Results. The effect of 2.4×10^{-7} M β-BuTX on the rat phrenic nerve-hemidiaphragm preparation is shown in figure 1. Control β-BuTX treatment caused a progressive decrease in the force of contraction evoked by phrenic nerve stimulation. A complete irreversible block occurred after approximately 80 min (A). Pre-incubation of β-BuTX for 60 min with 2.4×10^{-5} M manoalide delayed the onset and decreased the magnitude of the toxin-induced paralysis; manoalide sometimes caused an increase in muscle tone, shown as an increase in the base line B. Table 1 summarizes the results of a series of experiments in which the concentration of manoalide was varied. Statistically significant increases in the TI_{50} occurred at drug concentrations of 0.6, 1.2 and 2.4×10^{-5} M. At the highest concentration, no significant decline in twitch amplitude occurred over the entire observation period, indicating near complete inactivation of the toxin.

Table 2 is a comparison of the pre-incubation times required to inactivate this toxin. As can be seen, increasing the pre-incubation time of manoalide with β-BuTX progressively prolonged the TI_{50}. Mepacrine, a previously reported PLA_2 inhibitor[17, 19, 20], was without effect. The latter compound was tested at concentrations of 4.2×10^{-5} M, 8.4×10^{-5} M and 1.7×10^{-4} M with pre-incubation times of up to 2 h without producing any significant evidence of β-BuTX inactivation.

The apparent inactivation of β-BuTX prompted us to determine if manoalide would also inactivate purified PLA_2 since the presence of this enzyme as a subunit in β-BuTX has been implicated in its toxicity[7]. When manoalide was pre-incubated for 1 h with purified PLA_2, the subsequent hydrolysis of phosphatidylcholine was impeded. Figure 2 represents the graphic analysis of these results. When the percent inhibition of reaction velocity is plotted against manoalide concentration (fig. 2A), a typical saturation effect is seen. In all experiments with this source of PLA_2, manoalide inactivation uniformly plateaued at 75% inhibition of initial velocity. This raises the possibility that manoalide does not completely inactivate the enzyme. A double reciprocal plot of these data (fig. 2B) proved to be linear implying the presence of a homogeneous population of receptors. The apparent K_D for manoalide (4.8×10^{-7} M) indicates that this compound is a potent inactivator of PLA_2.

Table 1. Inactivation of β-BuTX paralysis by manoalide (pre-incubation 1 h)

Concentration Manoalide $\times 10^{-5}$ M	β-BuTX $\times 10^{-7}$ M	n	Mean $TI_{50} \pm$ SE
0.0	2.4	(4)	36.0 ± 4.2
0.6	2.4	(4)	45.2 ± 7.0*
1.2	2.4	(4)	75.7 ± 16.5*
2.4	2.4	(4)	137.0 ± 22.2*

* Statistically significant difference relative to β-BuTX alone, $p < 0.05$ unpaired Students' t-test.

Table 2. Effect of incubation time on β-BuTX** inactivation

Pre-incubation time (min)	n	Mean $TI_{50} \pm$ SE Manoalide 2.4×10^{-5} M	n	Mepacrine 2.1×10^{-5} M
0	(3)	64.60 ± 6.03	(2)	43.50 ± 2.50
25	(4)	83.20 ± 6.65*		
30	(5)	79.10 ± 3.46*	(2)	49.00 ± 4.00
60	(7)	140.50 ± 18.90*	(3)	46.90 ± 7.54
120	(5)	156.60 ± 22.50*	(3)	35.66 ± 4.67

* Statistically significant difference relative to controls (zero), $p < 0.5$ unpaired Students' t-test; ** 5 µg/ml β-BuTX.

Several experiments (data not shown) were undertaken to determine whether manoalide could act by 1. altering the incubation solution, 2. competing with β-BuTX for receptor sites on the nerve-muscle preparation or 3. reacting with the synthetic substrate, phosphatidylcholine. Prior treatment of the diaphragm with 2.4×10^{-5} M manoalide for 1 h before toxin exposure did not alter the TI_{50} of β-BuTX. When manoalide was added to the preparation after β-BuTX exposure, paralysis was also unimpeded. Identical results were obtained in experiments with purified PLA_2 and phosphatidylcholine, that is, manoalide reacts only with the enzyme and inactivation is dependent on pre-incubation.

Discussion. The experimental evidence reported here suggests that manoalide is a prototype for a new class of pharmacological probes. Inactivators of PLA_2 are relatively few, manoalide being the first structure of this type identified as having this pharmacological property. The only other compounds generally known to inactivate PLA_2 are mepacrine, p-bromophenacyl bromide (BPB) and some of its analogs[8]. BPB was first reported to inactivate pepsin[9] and was subsequently found to inactivate phospholipase A_2 from pancreatic tissue[10,11] and numerous snake venoms[8,12–15]. BPB is a halogenated analog of a series of hydrophobic reagents related to the tear gases[16]. It is structurally and pharmacologically unrelated to manoalide.

Mepacrine is an anti-malarial agent that decreases release of arachidonic acid in experimental models[17–19]. However, this compound will not inactivate PLA_2 purified from *Vipera ruselli*[20]. Furthermore in our studies, mepacrine did not inactivate the neurotoxic action of β-BuTX in concentrations as high as 10^{-3} M. Thus, based on these studies, the reported PLA_2 inhibiting properties of mepacrine may be due to mechanisms other than direct inactivation of the enzyme.

The observation that manoalide inactivates β-BuTX neurotoxicity and inactivates up to 75% of purified PLA_2 catalyzed hydrolysis of phosphatidylcholine suggests that this compound may react with a molecular sub-unit common to several toxins and other sources of PLA_2.

This compound could prove to be a useful probe to characterize the various forms of PLA_2 in nature and to help elucidate the role of these enzymes in various cellular processes.

Current experiments are under way exploring the mechanism(s) of PLA_2 inactivation. The anti-inflammatory properties of manoalide have been reported elsewhere[5].

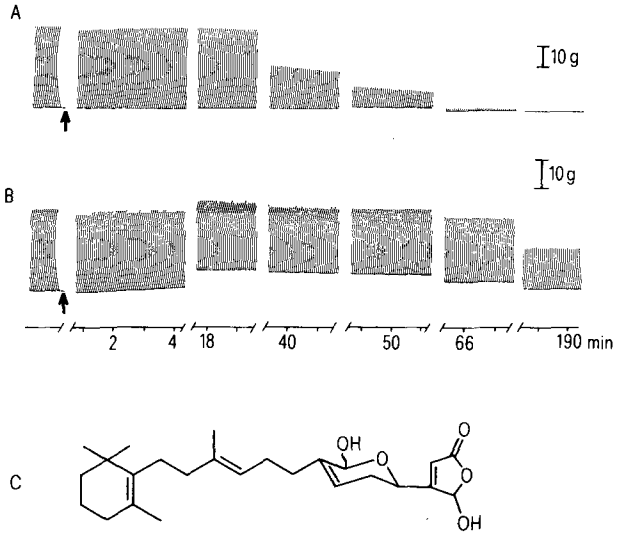

Figure 1. Effects of manoalide on prolonging the onset of β-BuTX induced neurotoxicity in the isolated rat phrenic nerve-hemidiaphragm. *A* Sample segment taken from continuous recordings of muscle twitch over 190 min show irreversible neuromuscular blockade by 2.4×10^{-7} M β-BuTX. *B* Comparable samples of a recording showing a delayed and decreased neuromuscular block after pre-incubation of β-BuTX with 2.4×10^{-5} M manoalide. *C* Chemical structure of manoalide.

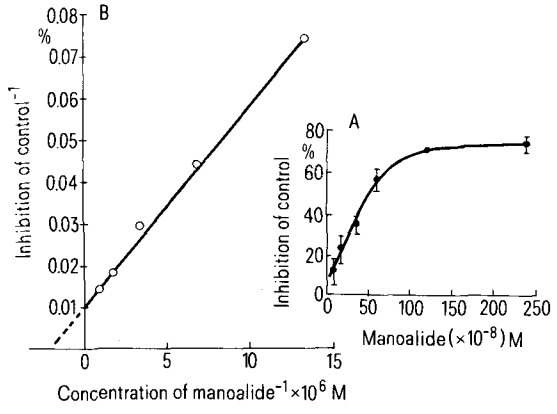

Figure 2. Manoalide inactivation of purified phospholipase A_2. Dose-response curve (n = 6). *A* Percent inhibition of rate of hydrolysis versus manoalide concentration. *B* Double reciprocal plot of % inhibition of rate of hydrolysis versus manoalide concentration. PLA_2 conc. = 0.33 units/ml.

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