EFFECTS OF MOJAVE TOXIN ON RAT SKELETAL MUSCLE SARCOPLASMIC RETICULUM

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Received July 9,1976

SUMMARY

Effects of the lethal fraction (MD-9) from the venom of the Mojave rattlesnake, Crotalus scutulatus, on sarcoplasmic reticulum were investigated. The calcium sequestering activity of the vesicles was reduced by the lethal fraction and subsequent release of calcium was enhanced. These effects were observed to be dependent upon MD-9 concentration and the length of preincubation time with the vesicles. An enhanced ATPase activity that was affected by concentration and MD-9 preincubation time was also observed. Both calcium uptake and ATPase activity effects may be due to a phospholipase activity associated with the fraction.

INTRODUCTION

Few North American rattlesnake venom proteins have been extensively purified and characterized. One species, the Mojave rattlesnake has sparked interest due to the acute lethal action of its venom. Several recent reports on lethal proteins from this venom have been published (1-3). The recent work of Bieber et al. (1) provided a lethal fraction from this venom which exhibited an acute cardiotoxic activity. Interest in the possible mode of action of this fraction led to the work presented here.

MATERIALS AND METHODS

Crude lyophilized Mojave rattlesnake venom was obtained from the Miami Serpentarium. DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals. Adenosine 5'-triphosphate (ATP) was obtained from Sigma Chemical Company. All other chemicals were of reagent grade quality.

The toxic fraction, MD-9, was isolated from crude Mojave rattlesnake venom by the method of Bieber, et al. (1).

Sarcoplasmic reticulum vesicles (SR) were isolated by differential centrifugation of rat skeletal muscle homogenates, following the procedure of Martonosi (4). The procedure was altered by omitting the second 0.6M KCl wash.

The SR pellets from the final centrifugation were suspended in 5mM histidine and 0.1M KCl at pH 7.3 to a protein concentration of 4-10mg/ml.

Phosphatidyl choline was prepared by extracting one egg yolk with 10 volumes of $CHCl_3$:CH_3OH (2:1, V/V). The extract was washed with 1-2 volumes of water to remove water soluble components. The organic phase was evaporated under reduced pressure and the residue was washed successively with 10ml of acetone and 10ml of chloroform. The lipid thus obtained was applied to a 1.6 x 21 cm silicic acid column that had been equilibrated with chloroform. Fractions were obtained by stepwise elutions with solvents of increasing polarity. Mixtures of chloroform and methanol were used in the following order: 150ml $CHCl_3$, 150ml $CHCl_3$: CH_3OH (4:1), 150ml (3:2), and 150ml (1:4). Fractions of 50ml were collected. The fraction containing a single spot identified as phosphatidyl choline was used for the phospholipase assays. Identification was made using comparative silicic acid thin-layer chromatography developed with $CHCl_3$:CH_3OH:32.5%$(NH_4)_2SO_4$ (51:21:3, V/V/V). Lipids were visualized by using the charring spray reagent of Ziminski and Borowski (5).

Protein was determined by the Lowry method (6) using BSA as a standard.

Calcium sequestering assays were modeled after the method of Madeira (7) in which the concentration of calcium in the assay media was measured using a Radiometer Calcium Selectrode adapted to an Instrumentation Laboratory expanded scale pH meter coupled to a Heath Built EUW-20A recorder. A bucking voltage device described by Madeira (7) was inserted between the pH meter and recorder. The assays were carried out at 25°C in 5ml of a 5mM Tris-HCl buffer at pH 7.0 containing 50mM KCl, 5mM $MgCl_2$, 60μM $CaCl_2$, 0.4-0.8mg SR protein, and 10μM ATP. The reaction was initiated by the addition of ATP. Lethal venom fraction, MD-9, was added at the concentrations indicated in the legends to the figures.

ATPase assays were performed using a Radiometer TTA31 microtitration assembly. The assay medium was identical to that used for the calcium sequestering assays with the ATP concentration increased to 0.1mM. The reaction was initiated by the addition of ATP and MD-9 was added as indicated. The ATPase activity was measured by the rate of addition of 0.0125N NaOH as titrant delivered from a 0.5ml syringe.

Phospholipase assays were performed using egg phosphatidyl choline as substrate in the medium reported by Salach *et al.* (8). The reaction was followed using the pH-Stat apparatus reported for the ATPase assays. Proton release was titrated with 0.025N NaOH. Egg phosphatidyl choline, solubilized in ethyl ether, was added to the reaction mixture to a final concentration of 7mg/ml. Crude venom and MD-9 were added to final concentrations as indicated in results.

RESULTS

The results of the calcium sequestering assays are presented in figures 1 and 2. The concentration dependence of MD-9 effects on calcium uptake is presented in figure 1. The SR was preincubated with MD-9 for 10 minutes prior to initiation of the reaction. The control was prepared by adding a volume of buffer equivalent to that of the toxin. The effect of preincubation time with MD-9 on calcium sequestering is given in figure 2. Assay conditions were

were monitored and were below 0.3 μM for Ca^{2+}, 0.07 μM for Mg^{2+} and Zn^{2+} was undetectable when using deionized-distilled water as the instrument blank.

Phosphate analysis were performed by the method of Bartlett (10), which was modified by adding 2 drops of 30% H_2O_2 to the digestion mixture at three 1 hr intervals after acid digestion to prevent the formation of charred carbonaceous material.

Rhodopsin concentrations were measured spectrophotometrically at 498 nm using a molar absorptivity of 41,000 (11). Protein concentration was measured at 280 nm and expressed as absorbance per ml.

Results and Discussion: The implication of Ca^{2+} as the mediator of photostimulation between ROS disc and the plasma membrane led us to characterize the native ion content of isolated retinal fractions. The Ca^{2+}, Mg^{2+} and Zn^{2+} compositions of samples of retinas, ROS and detergent extracted ROS are shown in Table I. These data demonstrate that ROS as well as detergent extracted ROS possess appreciable levels of all three cations relative to rhodopsin. At least a portion of the native metal ions are retained through the ROS isolation procedure and some ions are extractable with emulphogene.

Table I

Metal Ion Composition of Retinal Preparations

	Retina[1]	ROS Disc[2]		Emulphogene Extract
	µg atoms / g dry wt	µg atoms / g dry wt	g atoms / mole rhodopsin	g atoms / mole rhodopsin
Ca^{2+}	5.7	7.3	6.7	1.4
Mg^{2+}	37.2	3.8	3.6	1.4
Zn^{2+}	10.1	2.3	2.1	0.8

[1] Retinas and ROS were suspended in 50 mM PO_4 buffer and sonicated for 2 min. The solutions were refluxed for 3 hrs in 2 mM EDTA and 1% emulphogene, then centrifuged. Supernatant fluids were collected and diluted with deionized water. Divalent ions were determined as described in the text. Ca^{2+} and Mg^{2+} are determined in the presence of 1% Lanthanum oxide.

[2] 1.07 µmoles rhodopsin/g dry wt ROS.

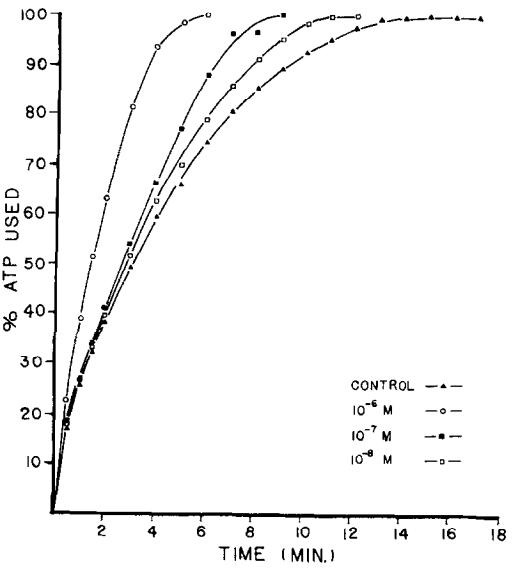

Figure 3. Effect of toxin concentration on SR vesicle ATPase activity. The reaction media contained 0.4mg of SR protein.

gated. Concentration dependent effects of MD-9 are presented in figure 3. Assay conditions and toxin concentrations are identical to those that were used for the calcium assays with the exception that ATP was added to a final concentration of 0.1mM. A constant preincubation time of 10 minutes was used. The pH-Stat reaction vessel was maintained at 25°C with a constant temperature circulating bath and a constant stream of humidified nitrogen was used to prevent evaporation and to exclude CO_2. Results are presented as the percent of total ATP used during the reaction time. The toxin did not exhibit any ATPase activity in the absence of SR. In figure 4, ATPase activities following fixed times of preincubation with 10^{-7}M toxin are presented. The control was prepared as a zero time preincubation using buffer instead of toxin.

Phospholipase assays were performed on the crude venom and MD-9 using egg phosphatidyl choline as substrate. The results of these assays appear in figure 5. For the control, buffer was added in place of the venom or MD-9 and incubation was allowed to proceed for a time period equivalent to that of the enzyme

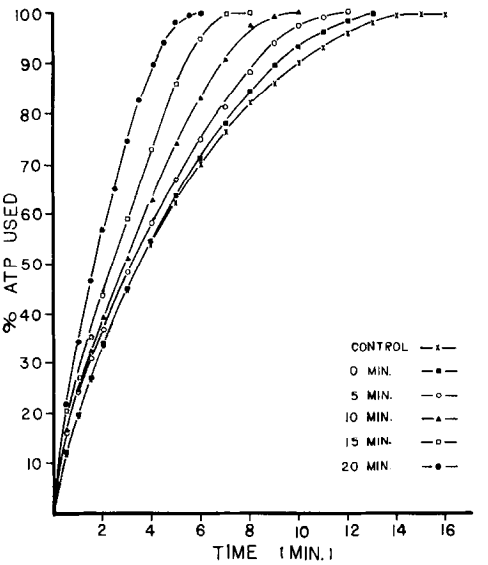

Figure 4. Effect of preincubation time with toxin on ATPase activity of rat skeletal muscle SR. The reaction media contained 0.4mg of SR protein.

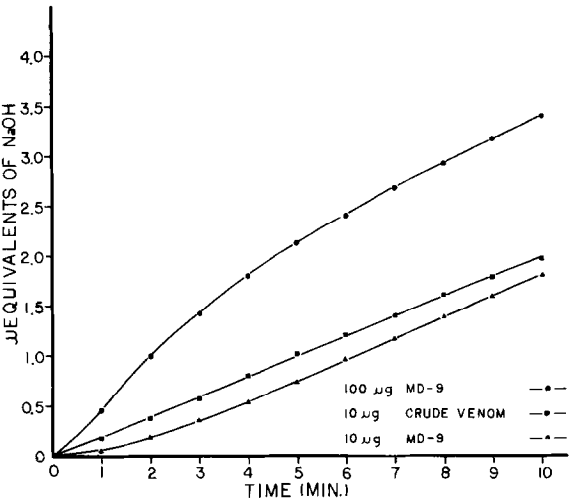

Figure 5. Phospholipase activity of crude venom and MD-9 with egg phosphatidyl choline as substrate. Assay conditions were: 0.5mM Tris-HCl, 0.5% Triton X-100, 20mM$CaCl_2$, 0.7lmM EDTA, 20mM NaCl, 0.01% BSA, pH 8.0, with 7mg/ml phosphatidyl choline. Total reaction volume was 2.0ml.

assays. The results are reported as μequivalents of NaOH used to titrate the protons that were released. The results have been corrected for autohydrolysis

of the lipids. Humidified nitrogen continuously purged the reaction vessel maintained at 25°C.

DISCUSSION

Calcium sequestration by rat skeletal muscle SR is affected dramatically by the toxic Mojave venom fraction, MD-9. As seen in figures 1 and 2, not only is there a concentration dependence but there is also a time dependence for the full effects of the toxin. The general nature of the effect appears to be a decrease in the uptake of calcium as well as an increased efflux of calcium from the vesicles. Such effects might reflect the formation of "leaky" vesicles and perhaps the presence of some inactive calcium binding sites. Results from unpublished membrane experiments designed to use chlortetracycline as a fluorescent probe as described by Lau et al. (9) support the data obtained with the ion selective electrode. The activity of ATPase increased when SR vesicles were treated with the toxin. These results can be accounted for by the formation of "leaky" membranes which might permit reversible binding and release of calcium by the transport system. Uncoupling of the ATPase and calcium pump might also account for the observed results. If the increased ATPase activity is due to an uncoupling effect, the lower initial calcium uptake observed in figures 1 and 2 might be expected. ATPase activity would also be increased if the toxin exposed reactive sites for ATP that were previously unavailable.

The evidence presented in figure 5 establishes a phospholytic activity for the lethal venom fraction, MD-9. This is consistent with the possibility of "leaky" vesicle formation. It should be noted that the activity of MD-9 on the lipids of SR in the ATPase assays was negligible. In ATPase assays performed without ATP additions less than 1% of the titratable protons for an ATPase assay were generated by MD-9 phospholytic activity. How the phospholytic activity is related to the toxicity of the MD-9 fraction, its significance to the SR effects, and explanations for the observed calcium uptake and ATPase effects are currently being investigated.

ACKNOWLEDGEMENTS

This work was supported in part by a fellowship from the Arizona Chapter of the American Heart Association.

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