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Effect of Portuguese Man-of-War Venom on Isolated Vascular Segments

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ABSTRACT

The purpose of this study was to characterize the mode of action of nematocyst venom from the Portuguese man-of-war (*Physalia physalis*) on isolated rabbit arterial ring segments, and to see if these *in vitro* effects were similar to those observed in the intact skeletal muscle vasculature of the dog (see Loredo *et al.*, J. Pharmacol. Exp. Ther. **232:** 301–304, 1985). The venom (0.021–4.28 μg/ml) produced dose-dependent relaxations of norepinephrine precontracted arterial segments from various vascular beds. Venom-induced relaxations were blocked by sodium meclofenamate (10–20 μg/ml), but not by atropine (6 μg/ml), propranolol (4–12 μg/ml) or quinacrine ($2–4 \times 10^{-5}$ M). These results were similar to those observed in the intact skeletal muscle vascular bed of the dog and further implicate the stimulation of endogenous prostaglandin synthesis as the mechanism by which *P. physalis* venom dilates vasculature.

The venom from Portuguese man-of-war (*Physalia physalis*) is known to be vasoactive (Burnett *et al.*, 1975; Loredo *et al.*, 1985). Its exact mode of action and identity of the active component is unknown. Previous experiments from our laboratory have implicated the role of the venom in the *de novo*, local synthesis of a vasodilatory PG or PG-like compound (Loredo *et al.*, 1985). Close i.a. injections of the venom into the circulation of skeletal muscle produce a dilation which is blocked completely by pretreatment with sodium meclofenamate, a cyclooxygenase inhibitor.

Questions unanswered by the *in vivo* experiments are: 1) whether this vasodilation is a function of some blood borne substance(s), or 2) of substance(s) endogenously produced by the vascular wall. The venom possesses a potent, toxic, hemolytic component (Tamkun and Hessinger, 1981) and hemolysis has been noted after repeated high doses of the venom *in vivo* (Loredo *et al.*, 1985). Whereas sodium meclofenamate would not block a K^+-mediated vasodilation from lysed erythrocytes, it might have an inhibiting effect on the synthesis of an eicosinoid-like compound by the blood. The purpose of these experiments was to see if the venom would affect an isolated vascular smooth muscle preparation in a similar manner as *in vivo*.

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Materials and Methods

General methods. Segments from the central ear, lingual, renal and distal femoral arteries (0.3–1.5 mm inside diameter) from 20 adult male California rabbits (2.0–2.7 kg) were removed quickly and placed immediately into chilled, aerated (95% O_2–5% CO_2) Krebs-Henseleit solution (Bevan, 1972). While still in the aerated physiologic solution, and with the aid of a stereomicroscope, the segments were cleaned of the surrounding tissue, taking care not to stretch them. A straight, 3-mm section of the artery, devoid of branches, was mounted on a device in which radial tension could be produced and measured (Bevan, 1972). The vessel was then placed into a constant-temperature organ bath of 15 ml of aerated Krebs-Henseleit, and allowed to equilibrate at 37°C for 60 min at a static tension of 300 mg before determining its tension characteristics and NE responsiveness. All vasoactive substances used in these experiments were made the morning of the experiments in Krebs-Henseleit solution. Tension was detected by a Grass model PT 3 strain gauge transducer (Grass Instrument Co., Quincy, MA), and the resultant signal was displayed continuously on a Grass model 7D polygraph.

P. physalis venom was prepared from isolated tentacle nematocysts, and assayed for toxicity and hemolysis activity by previously described methods (Tamkun and Hessinger, 1981), except that 25 mM acetate-TRIS buffer (pH 6.0) and 130 mM NaCl were used to extract the venom from the nematocyst capsules. The venom's i.v. mouse LD_{50} was 75 μg/kg, and the venom protein needed to cause half-maximal lysis ($K_{0.5}$) of washed rat erythrocytes was 25 ng/ml.

Calibration of vascular segments. Because we anticipated the venom would produce vascular relaxation, the segments had to be contracted to an optimum tension in order to demonstrate this relaxation. An optimum tension must be preset so that the contractile apparatus of the smooth muscle cell will react physiologically to the challenging doses of the various pharmacological agents used in these

ABBREVIATIONS: PG, prostaglandin; NE, norepinephrine.

experiments (Price *et al.*, 1981). Before evaluating the effect of the venom or its attempted blockade, the vascular segments had to be calibrated for tension development and NE responsiveness. With the segments at a tension of 300 mg (analogous to 100 mm Hg mean arterial blood pressure), a dose-response relationship of developed tension as a function of NE concentration (10^{-9} to 10^{-3} M) was established for each segment. The average NE concentration which produced 80% of the maximum vascular contraction (NE ED_{80}) was then used to determine the passive strain that resulted in the greatest tension development (L_{max}) in response to the agonist. This L_{max} was used as the optimum resting tension for the vascular segments in our experiments.

Experimental procedure. With the vessel segments at their L_{max}, and contracted with NE ED_{80}, the response to six concentrations (0.021–4.28 μg of venom per ml of bath solution) of the venom was determined. After the dose-response relationship of the venom was established, various pharmacologic blocking agents were tested for their ability to attenuate the activity of the venom. Before blockade, however, the response to a dose of 2.14 μg of venom per ml (analogous to 5 mg/kg b.wt. in the intact animal) was noted. Four blocking agents were used: 1) propranolol (4–12 μg of blocker per ml of bath solution), a *beta* adrenergic blocker; 2) atropine (6 μg/ml), a muscarinic blocker; 3) sodium meclofenamate (10–20 μg/ml), a cyclooxygenase inhibitor; and 4) quinacrine ($2–4 \times 10^{-5}$ M), a phospholipase A_2 inhibitor. The arterial segments were first preincubated with a blocking agent (atropine or propranolol, 30 min; sodium meclofenamate or quinacrine, 60 min). The effectiveness of the blocking agent was assessed by challenging the vessel segments with the following agonists or agonist precursor: 1) isoproterenol (80 μg/ml), for adrenergic receptors; 2) acetylcholine (10^{-5} M), for muscarinic receptors; and 3) arachidonic acid (40 μg/ml), a cyclooxygenase substrate. If the blocker did not abolish the challenge of the agonist, then additional blocker was used. When effective blockade was determined, the venom (2.14 μg/ml) was administered and its response compared with the preblocked condition.

Data analysis. Statistical significance was determined by a 2-way analysis of variance in conjunction with Duncan's new multiple-range test (Steel and Torrie, 1960). Curves were fitted to the data by the use of a second degree polynomial, least-squares technique (Creative Computing, Morristown, NJ). The data points for the curves were calculated by an Apple II+ personal computer (Apple Computer, Cupertino, CA) and plotted by a HiPlot X-Y plotter (Huston Instruments, Austin, TX).

Results

P. physalis venom caused relaxation of contracted, non-blocked vessel segments in a dose-dependent manner (fig. 1). Relaxation, induced by the venom, persisted until it was removed from the medium. The extent of venom-induced relaxation varied directly with the concentration of the venom in a sigmoid relationship (fig. 2). Renal segments appeared to be most sensitive to the venom, displaying relaxation at lower concentrations than the other segments. Maximum relaxation was achieved at a lowest concentration by the distal femoral segments; whereas, higher concentrations were necessary for maximum effect in renal and central ear segments. A maximum response was not reached by the lingual segments at the highest concentration used. The venom concentrations which produced 50% of the maximal effect (venom ED_{50}) were 0.26, 0.48 and 0.60 μg/ml for renal, distal femoral and central ear segments, respectively.

Pretreatment of the segments with atropine, propranolol or quinacrine did not attenuate the relaxation caused by the venom, but tended to enhance the relaxation (fig. 3), although not significantly ($P > .05$). Sodium meclofenamate, however, significantly decreased the venom's effect an average of 60%

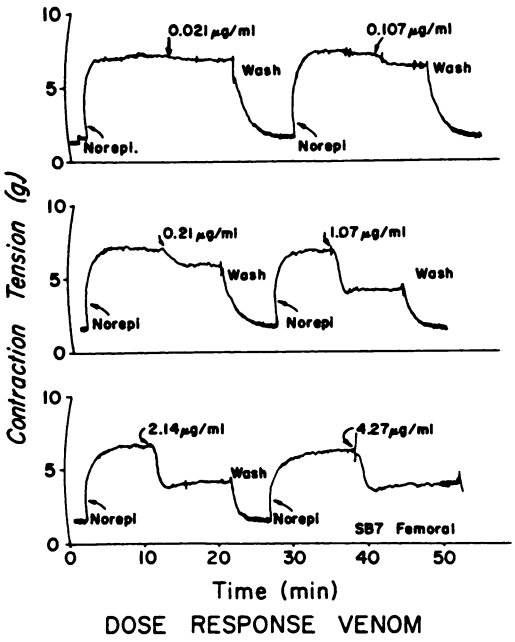

Fig. 1. A representative polygraph tracing of the action of *Physalia physalis* (Portuguese man-of-war) venom on an *in vitro* arterial ring segment preparation. The norepineprhine (Norepi) NE ED_{80} was used to contract the vessel segment. The effect of the venom was to produce relaxation of the NE-induced contraction in a dose-related manner.

($P < .01$), achieving complete blockade in a number of segments.

Discussion

The object of these experiments was to determine if nematocyst venom from Portuguese man-of-war could relax isolated arterial segments and, if so, whether the relaxation could be reversed by inhibiting PG synthesis. These two questions arose from our previous observations that man-of-war venom induces a sodium meclofenamate-inhibitable vasodilation of the skeletal muscle vascular bed in the intact dog. It also related to our hypothesis that this effect is mediated by a venom-induced synthesis of a PG-like substance (Loredo *et al.*, 1985). To test this hypothesis we evaluated the vasoactivity of the venom on isolated, small, blood vessel segments, by directly recording vascular wall tension in the presence and absence of specific blocking agents and inhibitors.

Our data show clearly that the venom produces vascular relaxation which can be blocked by inhibiting PG synthesis. These observations complement the results of experiments done on the intact dog, in which we showed that the i.a. injection of the venom into the skeletal muscle circulation produced vasodilation that was blocked completely by sodium meclofenamate, a cyclooxygenase inhibitor (Loredo *et al.*, 1985).

The venom-induced relaxation in the present study persisted so long as the venom was in contact with the vascular segment, contrary to the transient, venom-induced vasodilation observed in the intact dog. Binding of the venom's active component to the vessel segment appeared to be of low affinity, as at even the highest concentrations, *in vitro* vascular relaxation could be reversed with one change of the bathing medium. In contrast higher concentrations of NE required several changes of the medium to reverse its effects. It is unlikely that the transient vascular effects of the venom *in vivo* are due to the presence of

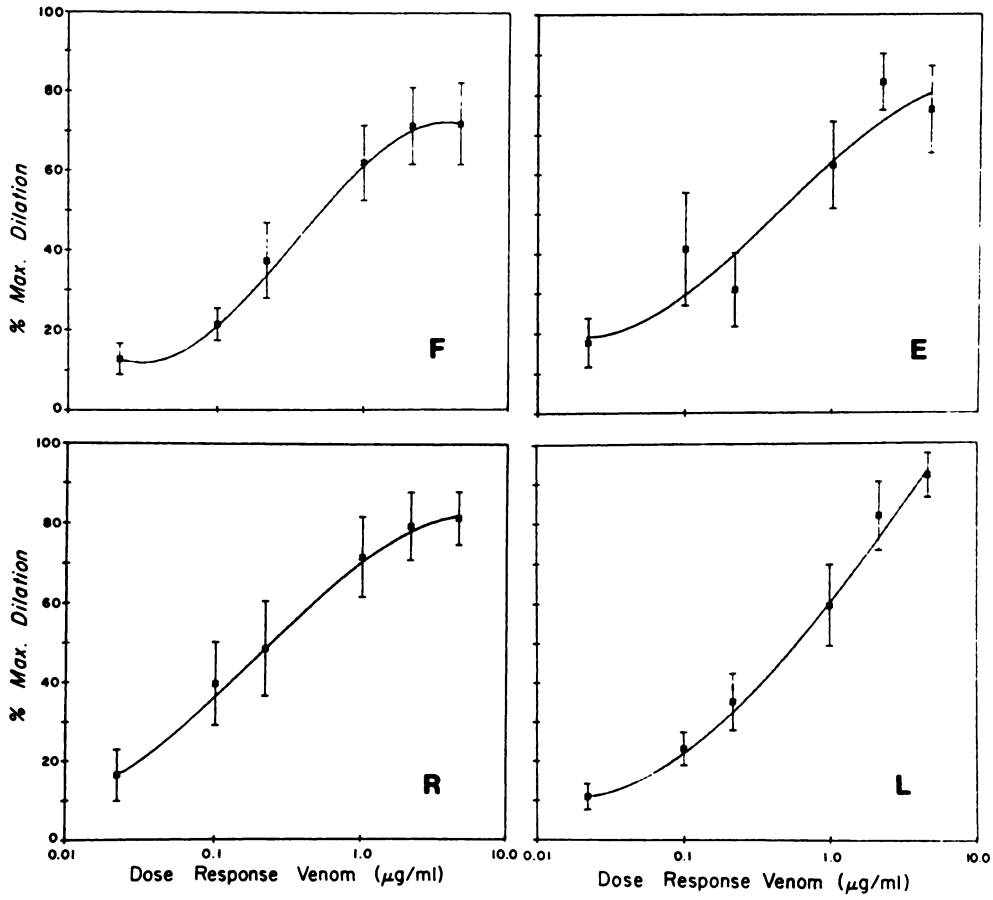

Fig. 2. *Physalia physalis* venom dose-response curves for precontracted (NE ED_{80}), isolated, renal (R), distal femoral (F), central ear (E) and lingual (L) arterial ring segments. Each graph point represents mean ± S.E.M. $N = 9$ for each graph. Max, maximum.

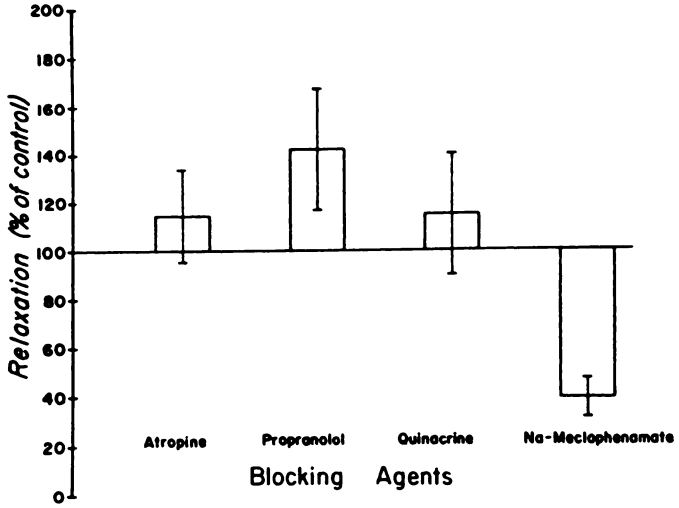

Fig. 3. Effect of *Physalia physalis* venom on the *in vitro* arterial ring segments, precontracted with NE ED_{80}, after pretreatment with the pharmacological blockers. Sodium meclofenamate significantly inhibited the action of the venom ($P < .01$). Each column represents mean percentage of difference in relaxation from the venom control ± S.E.M. (venom control relaxation would be 100%). $N = 6$, 10, 7 and 18 for the columns from left to right.

substances in the blood which quickly inactivate the venom, as higher concentrations of the venom injected i.a. in the intact dog resulted in mild systemic hypotension (Loredo *et al.*, 1985). This mild, but transient, systemic hypotension indicates that the venom remains active long enough to be recirculated through the animal. Its metabolic fate in blood, however, remains unknown. It is possible that the venom accomplishes its vascular action by modulating and/or stimulating an unstable vasoactive substance. Thus, the venom may act *in vitro* by continuously stimulating the synthesis of an unstable vasoactive agent, thereby producing a sustained relaxation. *In vivo*, due to the low binding affinity of the venom's active component, the circulation may dilute and carry this unstable substance away from the vascular wall giving a transient vasodilatory effect.

Sodium meclofenamate was the only blocking agent tested which significantly decreased the venom-induced relaxation, yet this blockade was not complete in all cases, as it was in our previous *in vivo* experiments. We believe this is due to the difficulty we had in determining, by titration, the meclofenamate concentration needed to block the challenging agonist. Inasmuch as the effective blocking concentration of sodium meclofenamate is close to the toxic dose, a complete blocking concentration was not always achieved in an effort to spare the vessel segment from damage.

Shier (1980) showed that Portuguese man-of-war venom induces high levels of membrane phospholipase A_2 activity and increases PG biosynthesis in cultured mouse fibroblasts. Even though the venom does not contain a phospholipase (Burnett and Calton, 1977; Tamkun and Hessinger, 1981), contrary to an earlier report (Stillway and Lane, 1971), it does contain an extremely potent and fast acting hemolytic protein (Tamkun and Hessinger, 1981). Thus, the venom may either act directly on vascular tissue to stimulate endogenous phospholipase A_2, or indirectly as an ionophore for some ion (*e.g.* Ca^{++}) which activates endogenous phospholipase A_2. On the other hand the

observation that quinacrine, a weak inhibitor of endogenous phospholipase A_2 activity (Feuerstein *et al.*, 1980; Salmon, 1982), did not affect the action of the venom implies that the venom does not stimulate PG production through the direct activation of phospholipase A_2 in vascular tissue.

Our data, therefore, indicate that man-of-war venom is a vasodilatory agent of vascular tissue. The mechanism of the action of venom does not seem to involve direct interaction with muscarinic or *beta* adrenergic receptors, or does it appear to affect adrenergic receptor sensitivity irreversibly. Rather, the results from these studies suggest that the vasodilatory action of the venom is mediated through the stimulation of PG synthesis by way of the cyclooxygenase pathway, and/or stimulation of the PG receptors in vascular smooth muscle. At this point our data do not support the hypothesis of the stimulation of phospholipase A_2 by the venom as a primary mechanism of action.

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References

BEVAN, J. A. AND OSHER, J. V.: A direct method for recording tension changes in the wall of small vessels *in vitro*. Agents Actions **2:** 257–260, 1972.

BURNETT, J. W. AND CALTON, G. J.: The chemistry and toxicology of some venomous pelagic coelenterates. Toxicon **15:** 177–196, 1977.

BURNETT, J. W., CALTON, G. J., MEIER, H. AND KAPLAN, A. P.: Mediators present in the nematocyst venoms of the sea nettle, sea wasp and Portuguese man-O'-war. Comp. Biochem. Physiol. **510:** 153–156, 1975.

FEUERSTEIN, G., DI MICCO, J. A. AND KOPIN, I. J.: Effect of indomethacin and quinacrine on blood pressure and plasma catecholamines in endotoxic shock (Abstract). Circ. Shock **7:** 196, 1980.

LOREDO, J. S., GONZALEZ, R. R., JR. AND HESSINGER, D. A.: Vascular effects of *Physalia physalis* venom in the skeletal muscle of the dog. J. Pharmacol. Exp. Ther. **232:** 301–304, 1985.

PRICE, J. M., DAVIS, D. L. AND KNAUSS, E. B.: Length-dependent sensitivity in vascular smooth muscle. Am. J. Physiol. **241:** H557–H563, 1981.

SALMON, J. A.: Inhibition of arachidonic acid metabolism. *In* Cardiovascular Pharmacology of the Prostaglandins, ed. by A. G. Herman, P. M. Vanhoutte, H. Denolin and A. Goossens, pp. 7–22, Raven Press, New York, 1982.

SHIER, W. T.: Activation of self-destruction as a mechanism of action for cytolytic toxins. *In* Natural Toxins, ed. by D. Eaker and T. Wadstrom, pp. 193–200, Pergamon Press, New York, 1980.

STEEL, R. G. D. AND TORRIE, J. H.: Duncan's new multiple range test. *In* Principles and Procedures in Statistics, pp. 107–109, McGraw-Hill Book Co., Inc., New York, 1960.

STILLWAY, L. W. AND LANE, C. E.: Phospholipase in the nematocyst toxin of *Physalia physalis*. Toxicon **9:** 193–195, 1971.

TAMKUN, M. M. AND HESSINGER, D. A.: Isolation and partial characterization of a hemolytic and toxic protein from nematocyst venom of the Portuguese man-of-war, *Physalia physalis*. Biochim. Biophys. Acta **667:** 87–98, 1981.

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