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Portuguese Man-of-war (*Physalia physalis*) venom induces calcium influx into cells by permeabilizing plasma membranes

Lincoln Edwards, David A. Hessinger*

Department of Physiology and Pharmacology,School of Medicine, Loma Linda University, Loma Linda, CA 92350, USA

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Abstract

Portuguese Man-of-war (*Physalia physalis*) nematocyst venom dose-dependently stimulates calcium ($^{45}Ca^{2+}$) influx into L-929, GH_4C_1, FRL, and embryonic chick heart cells. Venom-induced calcium influx is not blocked by ouabain, vanadate, nor organic calcium channel blockers, but is blocked by transition metal cations, such as lanthanum and zinc. Venom-induced calcium influx is accompanied in a dose-dependent manner by the release of intracellular lactate dehydrogenase, indicating a loss in plasma membrane integrity and cytolysis. Concentrations of zinc that block $^{45}Ca^{2+}$ influx also block lactate dehydrogenase release. Lanthanum, which also blocks $^{45}Ca^{2+}$ uptake, does not neutralize the cytolytic activity of the venom, but rather inhibits the venom's cytolytic action at the level of the target cell plasma membrane. Our findings indicate that Man-of-war venom causes an influx of calcium into several different cells types, not just those of the cardiovascular system, and this influx likely occurs by permeabilizing the plasma membranes of cells.

Keywords: Cultured embryonic chick heart cells; L-929 cells; GH_4C_1 cells; FRL cells; Portuguese Man-of-war; Nematocyst; Venom; Calcium influx; *Physalia physalis*; Nematocyst; Cytolysis

* Corresponding author. Tel.: +1-909-824-4564; fax: +1-909-478-4119.
E-mail address: dhessinger@som.llu.edu (D.A. Hessinger).

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1. Introduction

The fishing tentacles of the benthic siphonophore, the Portuguese Man-of-war (*Physalia physalis*) are armed with numerous stinging organelles called nematocysts (Cormier and Hessinger, 1981; Hessinger and Ford, 1988). Nematocysts are venomous, eversible, secretory products of complex effector cells called cnidocytes, found in all cnidarians. Suitable stimulation of the cnidocyte induces the nematocyst to rapidly evert its internal tubule (Holstein and Tardent, 1984). The nematocysts of the Portuguese Man-of-war are used to capture prey, such as fish and squid (Purcell, 1984) by means of injecting a potent venom. Man-of-war nematocyst venom is lethal to animals (Lane, 1960; Hessinger, 1988) and to humans (Stein et al., 1989). The major protein component of the venom is physalitoxin, which is a potent hemolysin (Tamkun and Hessinger, 1981).

Man-of-war venom has profound effects on the cardiac system of animals (Hastings et al., 1967), causing changes in rhythm (Garriott and Lane, 1969) and in ECG recordings (Larsen and Lane, 1966). On isolated rabbit atria the most pronounced effect is positive inotropy (Bonlie et al., 1988), which is directly proportional to the extracellular calcium level. The venom produces a seven-fold (or greater) increase in $^{45}Ca^{2+}$ influx into cultured embryonic chick heart cells by a mechanism not likely involving endogenous L-type or T-type calcium channels (Edwards et al., 1999).

From the literature, it appears that the in vivo actions of Man-of-war venom are directed primarily at the cells of the cardiovascular system, and the heart, in particular. At present, the mechanism of action of Man-of-war venom on cardiac cells is unknown. Alam and Qasim (1996) reported effects of venom fractions from *Physalia utriculus* on the tissues of several different organs. Their study, however, was a postmortem examination of envenomated animals and some of the effects observed by the authors may have been due to indirect effects, such as effects on the cells of the blood or on the blood vessels supplying these organs. Thus, it is not known if cell types other than those of the cardiovascular system are directly susceptible to the action of Man-of-war venom.

In this study, we report that Man-of-war venom increases $^{45}Ca^{2+}$ influx into several different types of cultured cell, including three types that are not of cardiovascular origin. Furthermore, the venom-induced calcium influx correlates with cytolysis as measured by the release of cytoplasmic lactate dehydrogenase. Venom-induced calcium influx and cytolysis are similarly inhibited by transition metal cations, further suggesting that the two effects are causally related.

2. Methods and material

2.1. Materials

Man-of-war venom was prepared from a single preparation of isolated nematocysts (Tamkun and Hessinger, 1981). L-929 cells and FRL cells were

obtained from Dr. Lora Green and from Dr. William Fletcher (Loma Linda VA Medical Center, Loma Linda, CA 92354, USA), respectively. GH_4C_1 cells were obtained from Dr. Armen Tashjian (Harvard School of Dental Medicine, Boston, MA). $^{45}Ca^{2+}$ was purchased from Dupont (Wilmington, DE). All other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Embryonic chick heart cell culture

Chick hearts were removed from 10-day old embryos and minced into fine pieces. Cold trypsin (0.25% w/v) was added to the minced heart tissue and placed on ice for 6 to 18 h to facilitate penetration of the tissue by the enzyme without damaging the cells. Excess trypsin solution was removed and the tissue was warmed to 37°C for 30 min to allow proteolysis of the extracellular matrix. A 1% antibiotic–antimycotic solution (10,000 units of penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B) was included in the medium. The diluted cell suspension was centrifuged at $600 \times g$ for 5 min. The cellular pellets were resuspended in culture medium containing 6% FCS, 40% medium 199, 53% balanced salt solution and 1% antibiotic–antimycotic solution, and then pooled. The pooled cells were incubated in a 75 cm^2 plastic culture flask at 37°C for 1 h to allow contaminating fibroblasts to adhere to the surface. The resulting supernatant heart cell suspension was decanted and then diluted in culture medium containing 6% FCS and plated at 0.5×10^6 cells/well in 24-well plates. The medium was changed once on the second day. Experiments were performed on the third day at which time a disperse monolayer of spontaneously beating heart cells had formed.

2.3. GH_4C_1 cell culture

Stock GH_4C_1 cells were grown in 75 cm^2 flasks as monolayers in Ham's F-10 medium containing 15% horse serum, 2.5% fetal bovine serum and 1% antibiotic–antimycotic solution at 37°C in a humidified atmosphere of 5% CO_2-95% air (Tashjian, 1979). Confluent cells were washed with Ca- and Mg-free Hanks solution and treated with Ca- and Mg-free Hanks containing 0.01% EDTA to detach the cells. Detached cells were collected and centrifuged at $600 \times g$ for 5 min and the supernatant discarded. The pelleted cells were resuspended in Ham's F-10 and plated into 24-well culture plates at 5×10^5 cells/well and incubated for four days before using.

2.4. L-929 fibroblast culture

L-929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic–antimycotic solution at 37°C in an atmosphere of 5% CO_2-95% air. Confluent cells were removed with 0.25% trypsin, centrifuged, and plated at a density of 5×10^5 cells per well and incubated for two days before using.

2.5. Fetal rat lung (FRL) cell culture

FRL cells were grown in DMEM containing 10% fetal bovine serum and 1% antibiotic–antimycotic solution at 37°C in an atmosphere of 5% CO_2-95% air. Confluent cells were removed with 0.25% trypsin, centrifuged, and plated at a density of 5×10^5 cells per well and incubated for two days before using.

2.6. Uptake of $^{45}Ca^{2+}$

Cultured cells (chick heart, GH_4C_1, L-929, and FRL) in 24-well plates were rinsed in HEPES buffered solution (HBS) (5 mM HEPES, 140 mM NaCl, 4 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.35) at 37°C for 5–10 min to remove old medium and to allow cells to adjust to the new medium. Next, the medium was replaced with $^{45}Ca^{2+}$ (4 μCi/ml) HBS with or without various concentrations of Man-of-war venom (or mellitin) and the cells were incubated at 37°C for 10 min. Cells were then briefly washed four times with cold HBS and solubilized with 1 ml of 0.1% sodium dodecyl sulfate (SDS). Aliquots were taken for scintillation counting and for protein assay by the BCA method (Smith et al., 1985).

2.7. Effects of ouabain and vanadate on $^{45}Ca^{2+}$ influxes

Cultured L-929 cells in 24-well plates were preincubated for 20 min with ouabain (1 mM) or vanadate (1 mM) in HBS or in HBS alone at 37°C. The solution was then removed and replaced with $^{45}Ca^{2+}$-containing HBS containing ouabain, vanadate, or neither with or without Man-of-war venom at 37°C for 10 min. Cells were then washed, solubilized, and aliquots taken for scintillation counting and protein assay.

2.8. Effects of metals on $^{45}Ca^{2+}$ uptake

Cultured L-929 cells were preincubated with La^{3+} or Zn^{2+} at concentrations ranging from 10^{-8} to 10^{-3} M in HBS at 37°C for 20 min. At the end of the preincubation, the medium was replaced with $^{45}Ca^{2+}$ HBS with or without Man-of-war venom in the presence of one or the other metals at 37°C for 10 min. Cultured cells were rinsed with HBS for 20 min and then treated with $^{45}Ca^{2+}$-HBS with or without Man-of-war venom at 37°C for 10 min. Venom preincubated with various concentrations of La^{3+} at 30°C for 20 min was diluted in $^{45}Ca^{2+}$-HBS and added to cells at a final venom concentration of 1 μg/ml for 10 min at 37°C. Cells were then washed and solubilized and aliquots taken for scintillation counting and protein assay.

2.9. Lactate dehydrogenase (LDH) release assay for cell lysis

Chick heart and L-929 cells in 24-well plates were preincubated with Zn^{2+} for 20 min then treated with or without venom (or mellitin) in HBS for 10 min at

37°C. After 10 min, cell-free supernatant fractions were assayed for lactate dehydrogenase (LDH) activity using a commercial kit (Sigma TOX-7; Decker et al., 1988) as per enclosed directions. The assay is based on the conversion of lactate to pyruvate in which NADH is generated to produce a colored formazan via a diaphorase-coupled reaction. The colored product is measured at 490 nm.

Briefly, a 300 μl aliquot containing equal volumes of LDH dye solution, assay substrate, and enzyme preparation was added to 600 μl of cell-free supernatant fractions from each well. The mixture was protected from light and allowed to sit at room temperature for 30 min, then the reaction was stopped with 90 μl of 1 M HCl and the absorbance read at 490 nm. In order to determine the maximum amount of LDH released, cultured cells were treated with 1/10 volume of LDH Assay Lysis Solution per well and incubated for 45 min at 37°C. Lysis was expressed as a percentage of the maximum.

2.10. Statistical analysis

Data were expressed as the mean ± standard error of the mean.

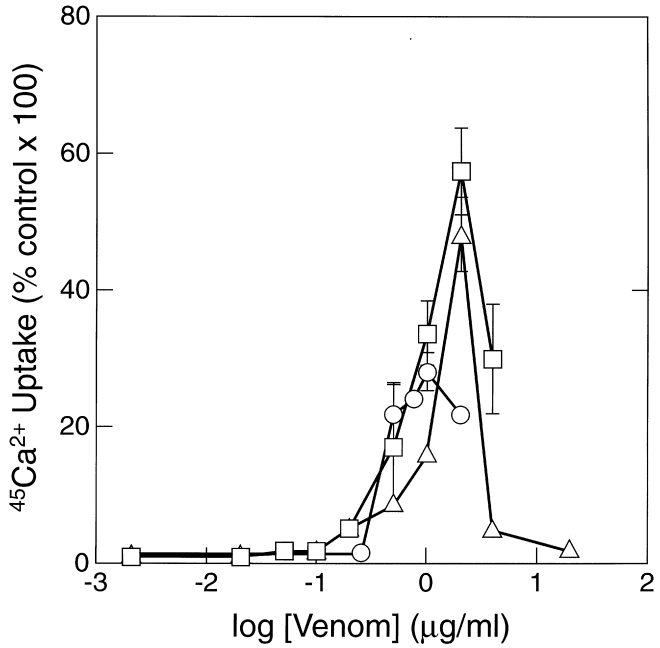

Fig. 1. Effect of Man-of-war venom on the calcium influx into different types of cultured cells. Cultured L-929 (square), GH_4C_1 (triangle) and FRL (circle) cells were incubated with HBS containing $^{45}Ca^{2+}$ at various concentrations of venom for 10 min and then assayed for $^{45}Ca^{2+}$ uptake. Each point represents the mean (±S.E.M.); $n = 6$.

3. Results

3.1. Ca^{2+} influx

Man-of-war venom increases $^{45}Ca^{2+}$ influx into different cell types, including GH_4C_1 rat pituitary cells, fetal rat lung cells (FRL), and L-929 fibroblasts (Fig. 1), in a biphasic, dose-dependent manner. Measurable $^{45}Ca^{2+}$ influx occurs only at doses above 0.1 μg/ml and is maximal at doses between 1.0 and 1.8 μg/ml in the different cell types. The maximal uptake into venom-treated L-929, GH_4C_1, and FRL cells are 5720, 4800 and 2800% of untreated control cells, respectively. The doses of venom producing half-maximal (EC_{50}) calcium uptake on L-929, GH_4C_1 and FRL cells are 0.76, 1.3 and 0.4 μg/ml, respectively. The effective range of venom concentrations for L-929 and GH_4C_1 cells is 0.1 to 1.8 μg/ml venom and for FRL cells the range is 0.23 to 1.0 μg/ml.

Since it has been proposed that Man-of-war venom affects heart by inhibiting Na^+/K^+-ATPase activity (Larsen and Lane, 1966), we tested vanadate (1 mM), a general ATPase inhibitor, and ouabain (1 mM), a blocker of the Na^+/K^+-ATPases, for the ability to block venom-induced $^{45}Ca^{2+}$ uptake. Vanadate does

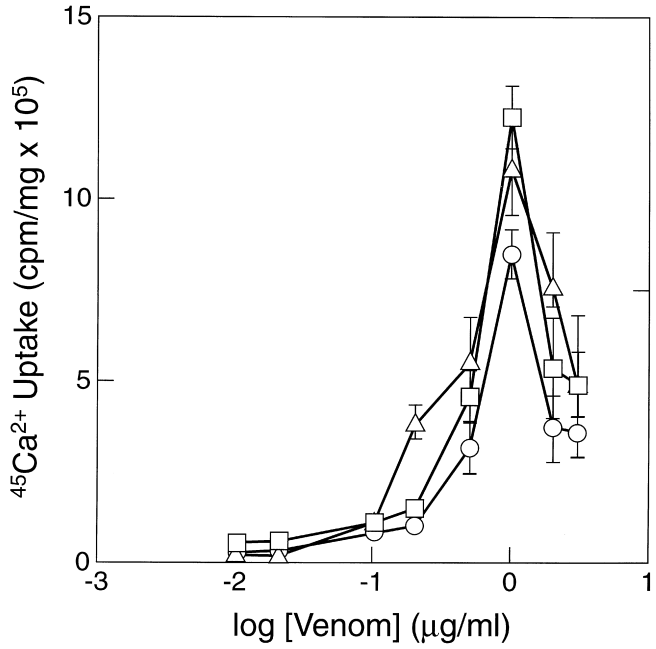

Fig. 2. Effects of ouabain and vanadate on venom-induced calcium uptake. L-929 cells were preincubated with ouabain (1 mM), vanadate (1 mM) or HBS for 20 min followed by addition of venom alone at different concentrations (square) or venom in the presence of ouabain (circle) or vanadate (triangle) for 10 min and then assayed for $^{45}Ca^{2+}$ uptake. Results are the means (±S.E.M.); $n = 6$.

not seem to affect venom-stimulated $^{45}Ca^{2+}$ uptake by L-929 cells and ouabain only affected $^{45}Ca^{2+}$ uptake at the 1 μg/ml venom dose (Fig. 2).

3.2. LDH release and cytolysis

Man-of-war venom causes the release of LDH, a cytoplasmic marker, from chick heart cells (Fig. 3) and from L-929 cells (Fig. 4) at doses of venom that also cause $^{45}Ca^{2+}$ influx. On chick heart cells the concentrations of venom at which half-maximal (EC_{50}) and maximal (EC_{100}) uptake of $^{45}Ca^{2+}$ occurs are 0.6 and 1.0 μg/ml venom, respectively, and the EC_{50} and EC_{100} values for LDH release are 0.5 and 1.0 μg/ml venom, respectively (Fig. 3). On L-929 cells, the EC_{50} for $^{45}Ca^{2+}$ uptake and LDH release are identical (0.55 μg/ml) and the EC_{100} values for uptake of $^{45}Ca^{2+}$ and LDH release are 0.75 and 1.0 μg/ml, respectively (Fig. 4). The similarities between the dose-response parameters for calcium uptake and cell lysis imply a common mechanism of action.

Mellitin is the major cytolytic component of honey bee (*Apis mellifera*) venom. On L-929 cells, mellitin causes a biphasic and dose-dependent ^{45}Ca uptake and a sigmoidal release of LDH (Fig. 5) similar to that of Man-of-war venom (Fig. 4). The EC_{50} and EC_{100} values for $^{45}Ca^{2+}$ uptake are 9 and 15 μg/ml, respectively.

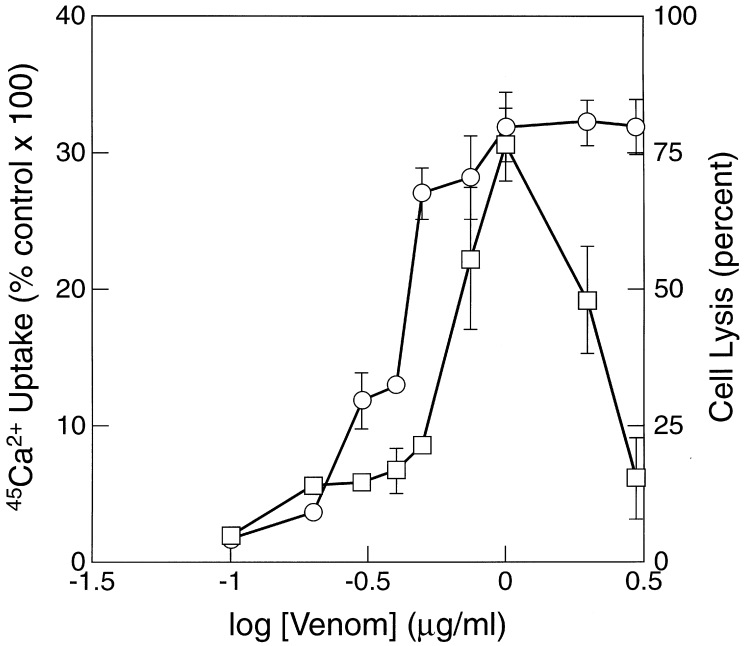

Fig. 3. Effects of Man-of-war venom on $^{45}Ca^{2+}$ uptake and LDH release (cell lysis) by embryonic chick heart cells. Cells were treated with venom in HBS (10 min, 37°C) and assayed for either $^{45}Ca^{2+}$ uptake (squares) or cell lysis (circles). Values are means (±S.E.M.); $n = 4$.

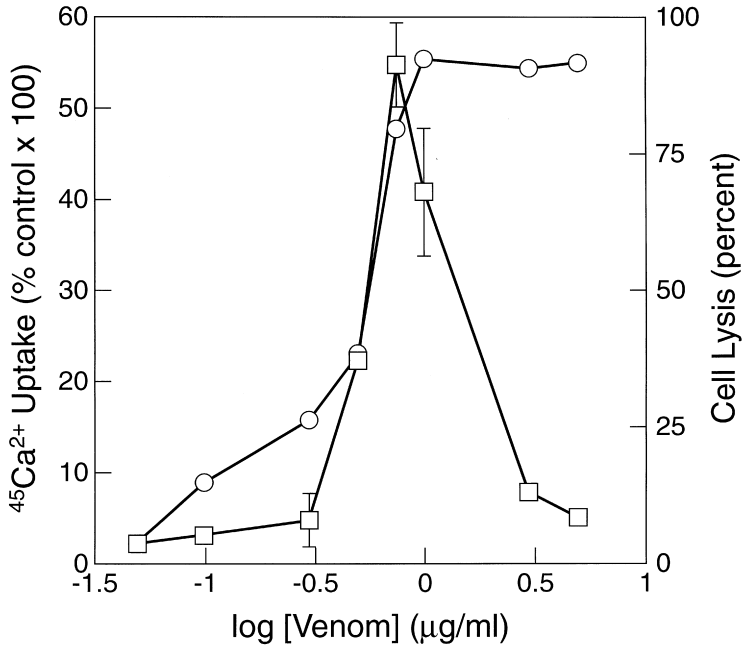

Fig. 4. Effects of Man-of-war venom on $^{45}Ca^{2+}$ uptake and LDH release by L-929 cells. Cells were treated with venom in HBS (10 min, 37°C) and assayed for either $^{45}Ca^{2+}$ uptake (squares) or cell lysis (circles). Values are means (±S.E.M.); $n = 4$.

Unlike Man-of-war venom, however, the dose of mellitin which causes half-maximal LDH release ($EC_{50} = 1$ μg/ml) is approximately one order of magnitude less than the dose which causes half-maximal $^{45}Ca^{2+}$ uptake.

In L-929 cells, both venom-induced $^{45}Ca^{2+}$ uptake and venom-induced LDH release are blocked by Zn^{2+} at concentrations greater than 1 μM (Fig. 6). The half-inhibitory doses (IC_{50}) of Zn^{2+} for both venom-induced calcium uptake and LDH release are the same (34 μM). Lanthanum also blocks venom-induced ^{45}Ca uptake into L-929 cells at concentrations greater than 10 μM with an IC_{50} of 32 μM (Fig.7). However, if Man-of-war venom is preincubated with lanthanum at different concentrations and then added to cells, inhibition of venom-induced $^{45}Ca^{2+}$ uptake is not observed (Fig. 7).

4. Discussion

Previous studies have shown that Man-of-war venom affects several components of the cardiovascular system, including the heart (Larsen and Lane, 1966; Hastings et al., 1967; Garriott and Lane, 1969), the vasculature (Loredo et al., 1985, 1986, Luo et al., 1990), the blood cells (Lin and Hessinger, 1979; Flowers and Hessinger, 1981; Tamkun and Hessinger, 1981) and cultured heart cells

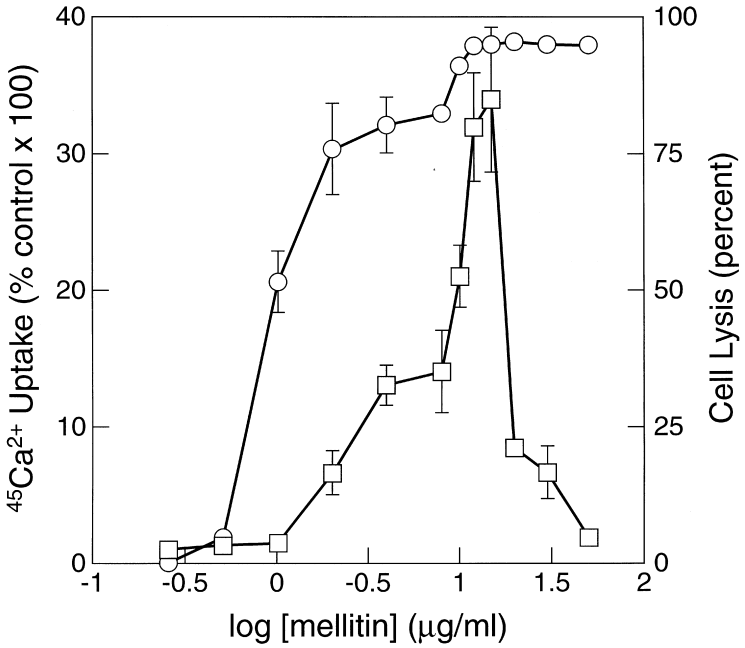

Fig. 5. Effects of mellitin on $^{45}Ca^{2+}$ uptake and LDH release by L-929 cells. Cells were treated with mellitin in HBS (10 min, 37°C) and assayed for either $^{45}Ca^{2+}$ uptake (squares) or LDH release (circles). Values are means (±S.E.M.); $n = 4$.

(Edwards et al., 1999). It is not known whether the venom specifically targets cells of the cardiovascular system cells or if cells of noncardiovascular origin are also susceptible.

In the present report, we show that three different, noncardiovascular cell types, GH_4C_1 rat pituitary cells, fetal rat lung cells (FRL) and L-929 fibroblasts, are susceptible to Man-of-war venom in much the same manner and at much the same dose as cultured embryonic chicken heart cells. Man-of-war venom biphasically stimulates calcium influx into all four cell types. Thus, this action of Man-of-war venom is not restricted to cells of the cardiovascular system.

We have previously shown that Man-of-war venom stimulates calcium influx into cultured embryonic chick heart cells by a mechanism not likely involving preexisting L-type or T-type calcium channels (Edwards et al., 1999). It had been suggested by Larsen et al. (1966) that Man-of-war venom blocks the Na^+/K^+-ATPase pump of heart tissue. We, therefore, investigated the action of ouabain, a blocker of the Na^+/K^+-ATPase, and vanadate, a generalized ATPase inhibitor, on Man-of-war venom-induced ^{45}Ca uptake. Our results show that neither ouabain nor vanadate block Man-of-war venom-induced ^{45}Ca uptake by L-929 cells, suggesting that Man-of-war venom-induced $^{45}Ca^{2+}$ uptake does not occur by a mechanism involving the Na^+/K^+-ATPase or other ATPases.

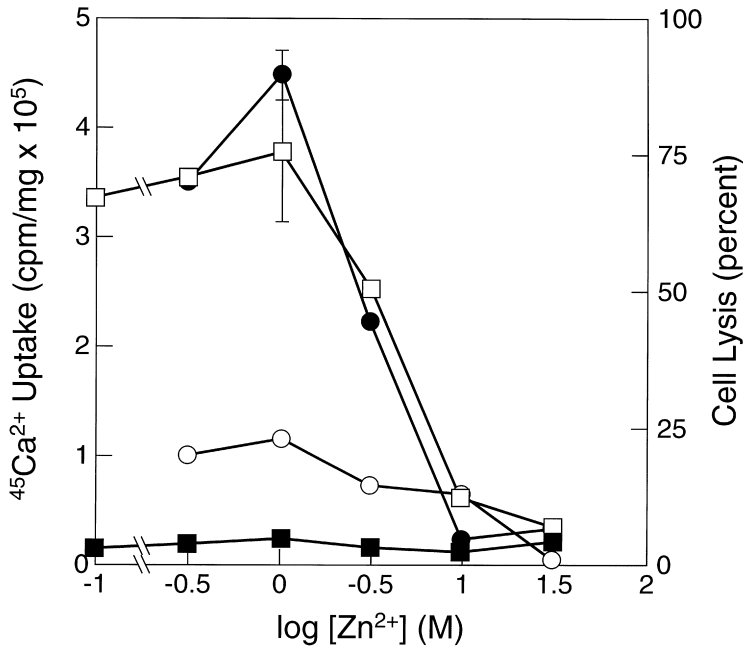

Fig. 6. Effects of Zn^{2+} on Man-of-war venom-induced $^{45}Ca^{2+}$ uptake and LDH release. L-929 cells were preincubated for 20 min in various concentrations of Zn^{2+} before adding $^{45}Ca^{2+}$ with (open squares) or without (closed squares) venom (1.0 μg/ml) in HBS containing Zn^{2+}. Adherent cells were washed and assayed for $^{45}Ca^{2+}$ uptake. Culture supernates from venom-treated (filled circles) and control (open circles) cells were assayed for cell lysis. Values are means (±S.E.M.); $n = 4$.

The action of Man-of-war venom on ionic fluxes across plasma membranes bear similarity to the actions reported of α-latrotoxin, maitotoxin, and mellitin. The α-latrotoxin from black widow spider venom (Frontali et al., 1976; Grasso et al., 1982; Nicholls et al., 1982) forms nonclosing ionic channels which are permeable to K^+, Na^+ and Ca^{2+} and insensitive to conventional Na^+ and Ca^{2+} channel blockers (Wanke et al., 1986). Because of its primary ionic effects, α-latrotoxin can also produce cell lysis. Conductances induced in black lipid membranes by α-latrotoxin are blocked by divalent cations in the order of potency: $Co^{2+} > Ni^{2+} > Zn^{2+}$ (Bhakdi et al., 1984). Like α-latrotoxin, Man-of-war venom causes an increase in membrane permeability to K^+, Na^+ and Ca^{2+}, which is insensitive to organic channel blockers (Edwards et al., 1999). However, the blocking potency of metal ions on Man-of-war venom-induced $^{45}Ca^{2+}$ uptake into chick heart cells is $Zn^{2+} > Ni^{2+} = Co^{2+}$ (Edwards et al., 1999), the reverse of the order of potency for α-latrotoxin. The action of α-latrotoxin is specific for neuronal cell types, whereas, as shown in the present report, Man-of-war venom appears to be nonspecific with respect to cell type.

Some of the effects of Man-of-war venom also resemble those of maitotoxin. Both maitotoxin (Sladeczek et al., 1988) and Man-of-war venom (Edwards et al.,

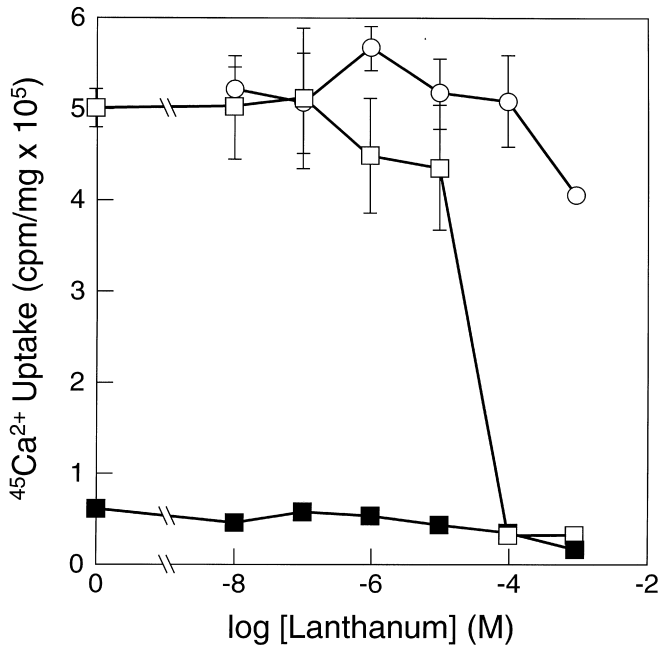

Fig. 7. Effects of La^{3+} on venom-induced $^{45}Ca^{2+}$ uptake. L-929 cells were preincubated for 20 min in different concentrations of La^{3+} before adding $^{45}Ca^{2+}$ with (open squares) or without (closed squares) venom (1.0 μg/ml) in HBS containing La^{3+}. Cells were also treated with venom (1.0 μg/ml; 10 min, 37°C) that was preincubated with La^{3+} for 20 min (circles). Values are means (±S.E.M.); $n = 6$.

1999) cause $^{45}Ca^{2+}$ uptake, K^+ efflux and Na^+ influx with the calcium influx and sodium influx being insensitive to conventional organic blockers. Zinc is the most potent divalent metal to block maitotoxin-induced $^{45}Ca^{2+}$ uptake, with an IC_{50} of 41 versus 34 μM for Man-of-war venom. Maitotoxin lyses BC_3H_1 cells (Sladeczek et al., 1988) and Man-of-war venom lyses chick heart cells (Fig. 3) and L-929 cells (Fig. 4) as indicated by venom-induced LDH release.

Mellitin, a cytolytic component of bee venom lyses all cells on which it has been tested (Weissmann et al., 1969; Haberman, 1972; Olson et al., 1974). Mellitin also forms voltage-dependent, anion-selective channels at low doses (Tosteson et al., 1985). We have shown that mellitin causes $^{45}Ca^{2+}$ uptake into L-929 cells in a biphasic manner similar to Man-of-war venom and, in addition, causes the release of LDH (Fig. 5). Unlike Man-of-war venom, the dose of mellitin that cause half-maximal LDH release ($EC_{50} = 1$ μg/ml) is only about one-tenth that which causes half-maximal $^{45}Ca^{2+}$ uptake ($EC_{50} = 9$ μg/ml). This suggests that mellitin causes calcium uptake and cell lysis by separate mechanisms, whereas Man-of-war venom likely causes calcium uptake and cell lysis by the same mechanism.

Known pore-formers, such as mellitin (Bhakdi et al., 1984), *Staphylococcal aureus* α-toxin (Muller-Eberhard, 1984), complement (Pasternak, 1987) and

hemolytic viruses (Bashford et al., 1986) exhibit steep cytolytic dose-responses, indicative of positive cooperativity among lytic proteins. The lytic effects of these proteins are also blocked by divalent metals, of which Zn^{2+} is one of the most potent, with IC_{50} values between 10 and 100 μM, as is Man-of-war venom on L-929 cells and chick embryonic heart cells.

The mechanism by which divalent metal cations inhibit cytolytic proteins remains obscure. For leukotoxin from *Actinobacillus actinomycetemcomitans*, it has been suggested that the metal cations promote closure of pores or channels formed by the leukotoxin (Iwase et al., 1990) and the same idea has been advanced to explain the inhibitory effect of Ca^{2+} and Zn^{2+} against porforin (Bashford et al., 1984). Complement-induced lysis of nucleated cells is inhibited by high extracellular calcium and calcium may increase the rate of removal of the lysin from the plasma membrane via endocytosis or by membrane shedding (Shin and Carney, 1988).

Lanthanum is also a potent inhibitor of several cytolytic toxins (Sladeczek et al., 1988; Iwase et al., 1990). In the present study, 100 μM lanthanum completely blocked venom-induced calcium influx into L-929 cells when the cells were preincubated with the metal (Fig. 7). However, when the venom was preincubated with 100 μM lanthanum and then added to the cells, no inhibitory effect was observed, suggesting that lanthanum exerts its inhibitory effect at the level of the plasma membrane and not upon the free venom proteins. We speculate that metals, such as La^{3+} and Zn^{2+}, inhibit Man-of war venom by preventing oligomerization of venom proteins in the target membrane by blocking expressed anionic sites needed for oligomerization.

Razin (1972) suggested that metals act on the phospholipids of the target membrane to decrease membrane fluidity. The change in membrane fluidity may interfere with the lateral diffusion and aggregation of cytolytic venom components to form pores or channels (Miyake et al., 1988). Lowering the temperature can also decrease membrane fluidity. The hemolysis caused by Man-of-war venom on rat erythrocytes is inhibited at lower temperatures (Tamkun and Hessinger, 1981).

In summary, Man-of-war venom increases $^{45}Ca^{2+}$ influx into various types of cultured cells, including embryonic cardiomyocytes, L-929 cells, FRL cells and GH_4C_1 cells. Venom-induced influx is neither blocked by ouabain, a blocker of the Na^+/K^+-ATPase pump, nor by vanadate, a general ATPase blocker. Cultured cells are also lysed by Man-of-war venom, as indicated by LDH release. Man-of-war venom lyses chick heart cells and L-929 cells at doses of venom ($EC_{50} = 0.55$ μg/ml) that also cause $^{45}Ca^{2+}$ uptake ($EC_{50} = 0.55$ μg/ml), suggesting a common mechanism. In addition, both processes are blocked by the same doses of Zn^{2+} (Fig. 6), an inhibitor of other cytolytic proteins (Avigad and Bernheimer, 1976). The venom also exhibits similarities to known pore-forming cytolysins, such as mellitin and bacterial cytolysins, by exhibiting steep dose-response curves. In conclusion, we hypothesize that Man-of-war venom causes $^{45}Ca^{2+}$ influx and cell lysis by permeabilizing the plasma membrane of target cells.

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