Molecular Mechanisms of Physalia Physalis Venom Modulation of Cardiac and Vascular Functions

Eva Luo

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Abstract

MOLECULAR MECHANISMS OF PHYSALIA PHYSALIS VENOM MODULATION OF CARDIAC AND VASCULAR FUNCTIONS

by

Eva Luo

Portuguese man-of-war (Physalia physalis) venom has profound effects on the cardiovascular system of man and animals. The venom produces vascular relaxation in norepinephrine precontracted, intact rabbit femoral arterial rings by stimulating vasodilatory prostaglandin synthesis in the endothelium. This action does not depend on endothelium-derived relaxing factor (EDRF). P. physalis venom produces a vascular constriction on KCl depolarized, intact rings. This is completely inhibited by dibenzyline and yohimbine. Increased membrane permeability to calcium may be involved in direct vascular smooth muscle stimulation. The venom has a positive inotropic effect on rat atrial preparations, which is intensified by increased extracellular calcium concentration. P. physalis venom markedly increases calcium influx of cultured embryonic chick heart cells in a dose dependent manner by activating L type calcium channels. The increased calcium influx is consistent with the positive inotropic action on the cultured heart cells observed under the microscope. This effect is completely inhibited by diltiazem and verapamil. Sodium influx of cultured embryonic chick heart cells is moderately increased (about one sixth of increased calcium influx) under the influence of P. physalis venom. Flecainide (a sodium channel blocker) and verapamil do not inhibit the increased sodium influx. The venom may accelerate sodium/calcium exchange directly and/or indirectly by increased calcium influx, the major mechanism for the embryonic chick heart to eliminate excessive cytoplasmic free calcium. Rubidium (an analog of potassium) influx is not affected by P. physalis venom at lower dose levels. A high concentration of the venom, nevertheless, decreases rubidium influx, implicating an inhibition of the Na+, K+-pump. Greatly increased contraction of the cultured heart
cells associated with a large calcium influx may result in energy depletion, hence, inhibition of the Na+, K+-pump. The increased calcium influx of the cultured heart cells appears to be highly correlated with an increased efflux of rubidium. Stimulation of calcium-dependent potassium channels is an indirect effect of the venom. Activation of ATP-sensitive potassium channels may also contribute to the increased rubidium efflux when ATP depletion occurs under the condition of greatly increased contraction in the presence of a high venom concentration. *P. physalis* venom does not affect 2-deoxy-D-glucose uptake by cultured embryonic chick heart cells. However, at extremely high doses the uptake of the glucose is reduced. Glucose transport and metabolism may be disturbed by the excessive cytoplasmic calcium caused by *P. physalis* venom.
MOLECULAR MECHANISMS OF PHYSALIA PHYSALIS VENOM MODULATION OF CARDIAC AND VASCULAR FUNCTIONS

By

Eva Luo

A Dissertation in Partial Fulfillment
of the Requirements for the Degree of Doctor of Philosophy
in Pharmacology

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Each person whose signature appears below certified that this dissertation in his opinion is adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy.

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INTRODUCTION

General Aspects of Physalia physalis

Portuguese man-of-war (Physalia physalis), a member of the phylum Coelenterata, is distributed in the tropical and subtropical Atlantic, and occasionally as far as the Bay of Fundy and the Hebrides (Halstead, 1978). The man-of-war is a floating colony composed of four specialized adult hydroids that perform specialized functions and share the task of survival. The float or pneumatophore is one entity; the fishing tentacles or the dactylozooids capture small fish and squid (Porcell, 1984) and convey them to the third kind, the gastrozooids that digest the food. The fourth kind carries out the task of reproduction (Lane, 1960a).

Floating in the sea, its natural environment, the float, a hollow crested bladder, may be as large as 12 inches long, 6 inches wide and 6 inches high, varying in color from blue, through azure, purple, lavender or orchid, to pink or scarlet. Although the float appears to drift with the wind, actually it makes a course at a significant angle to the surface wind. The boat-like shape and the sail-like crest of the float facilitate the downwind travel of the animal. The tentacles, acting as sea anchors under the floating body, have a stabilizing effect (Lane, 1960a).

The number of tentacles and their length vary with the size of P. physalis. These tentacles may extend to a length of 30 meters in deep water. The nematocysts, stinging organelles filled with venom, are contained within cnidocytes located in the superficial epithelium of the tentacle. There is an average of about 750,000 nematocysts in each tentacle (Halstead, 1978).

The individual nematocyst is a hollow spherical capsule. The capsular wall is inverted in at one point to form a long hollow inverted tube coiling into approximately 20 circles with nearly the same diameter inside the capsule. The tube is armed along one third its proximal length with barbs and spines. When the tubule rapidly everts during discharge these barbs are present on the outer surface of the tubule pointing backward towards the capsule. The lumen of the tubule where the tubule joins the
capsule is occluded with a plug-like operculum. In the everted confirmation the tubule extends fifty to seventy times the diameter of the parent capsule (Hessinger, 1988; and Tardent, 1988).

The origin of the force that propels the rapid eversion of the tubule is not clear. Three hypotheses have been proposed. The contractile hypothesis states that the pressure required for opening the operculum and the eversion of the tubule is generated by the contraction of either the cnidocyte cytoskeleton or the cyst wall. The intrinsic forces hypothesis postulates that the energy deployed during eversion stems from spring-like intrinsic forces which are built up during cnidogenesis and stored within the walls of the cyst and the tubule. During eversion they would be released like the energies stored in a tightened spring. The osmotic hypothesis argues that the intracapsular pressure increases immediately before and/or during the process of discharge by swelling of the cyst matrix (Tardent, 1988).

When the Man-of-War is moving through the water, the fishing tentacles undergo spontaneous rhythmic movements to sample the water constantly. If a prey organism is detected, the nematocysts are stimulated and trigger the immediate release of the coiled nematocyst thread-like tubule. When the tip penetrates the victim, the venom within the nematocyst capsule is conveyed directly into the body of the prey through the hollow tubule (Cormier and Hessinger, 1980).

Chemical and mechanical stimuli together trigger the discharge of nematocysts in sea anemones and in the Portuguese man-of-war (Parker and Alstyne 1932). Two groups of low molecular weight substances, amino acids and N-acetylated sugars have been identified to predispose or sensitize cnidocytes to triggering tactile stimuli that result in the discharge of nematocysts. In sea anemones N-acetylated sugars of mucins and chitins on the surface of the prey or amino acids leaking from the nematocyst-inflicted wounds of the prey activate chemoreceptors located on adjacent supporting cells. Second messengers generated from the ligand-receptor complex sensitize associated cnidocytes to mechanical stimuli originating
from the prey, to initiate discharge (Thorington and Hessinger, 1988).

*P. physalis* nematocysts appear to be independent effectors with little or no modulatory influence from accessory cells or the nervous system (Hessinger and Ford, 1988). In addition to prey, increased hydrostatic pressure, mechanical pressure and/or acidity may also set off discharge of the nematocysts. The magnitude of discharge of the nematocyst is proportional to the contact area between the tentacle and the prey (Lane, 1960a and 1960b).

**The Nematocyst Venom of *P. physalis***

Two size groups, 10.6 and 23.5 nm in diameter, of *P. physalis* nematocysts have been separated by flow cytometry (Burnett *et al.*, 1986) and by differential centrifugation (Tamkun and Hessinger, 1981). The smaller size was reported to be more toxic than the larger one. However, both size groups contain common proteins of 69,000, 82,000 and 50,000-65,000 daltons molecular weight.

Preparative gel electrophoresis (Burnett and Calton, 1974) and gel-filtration on Sephadex G-200 (Calton and Burnett, 1973a) have been used unsuccessfully to fractionate the crude venom. A monoclonal antibody to the lethal activity of *P. physalis* venom has been developed (Gaur *et al.*, 1982) and used in immuno-chromatography to purify cardiotoxins from *Chrysaora quinquecirrha* (sea nettle) polyps and cysts (Olson *et al.*, 1985) and from *Chironex fleckerii* (sea wasp) nematocysts (Olson *et al.*, 1984)

Lyophilized venom is stable for several years in evacuated capsules stored at -20 °C in the dark. The venom is thermolabile in solution, its activity being completely destroyed during 5 minutes at 60°C. Reaction with ethanol, acetone, petroleum ether, dimethyl ether, and most other organic solvents destroys the activity of the venom (Lane, 1960b).

Venom extracted from the nematocyst is a mixture of a variety of agents including indoles, kinins and different peptides (Soppe, 1989; and Lane, 1961). Elastases (Werb *et al.*, 1982), a DNA endonuclease (Neeman *et al.*, 1980), and a collagenase (Lal *et al.*, 1981) have also been found in
P.physalis venom. Stillway and Lane (1971) reported phospholipases A and B activities in the venom, but it has not been confirmed by later studies (Hessinger, 1988). A potent hemolytic and toxic protein, composed three subunits of equal size and containing 10.6% carbohydrates by weight, has been isolated. It is rod like in shape, and has a sedimentation coefficient of 7.8 S and a molecular weight of 240,000 daltons (Tamkun and Hessinger, 1981).

The Toxicity of P. physalis Venom

Lethality

P. physalis venom is one of the most toxic of all known venoms. The mortality curve of the venom has an extremely steep slope, thus there is a very narrow range between the maximal non-lethal dose and the minimal lethal dose. The LD50 for the nematocyst extract ranges from 32 to 2,0000 μg/kg owing to the degree of contamination in the extraction process (Hessinger, 1988). The LD50 in mice for the isolated and purified rod-like hemolytic protein is approximately 200 μg/kg (Tamkun and Hessinger, 1981). Twice the LD50 can kill injected mice in seconds (Hessinger, 1988), while others have reported that three LD50 doses killed mice in about 3 hours (Burnett et al., 1985).

General signs and symptoms

On the east coast of the United states it is common for ocean bathers to come in contact with the tentacles of an Atlantic Portuguese man-of-war. Although P. physalis venom is highly toxic it is only occasionally lethal to humans. Stings from the man-of-war result in a pricking sensation, edema, burning pain, and skin irritation with erythema or a purple discoloration in a whip-patterned distribution. A discontinuous line of papules rapidly coalesces to form a linear welt or a pattern of discrete wheals, which originates from different tentacles. There are subtle differences in the external appearance of each sting according to the species involved (Auerbach and Hays, 1987). Systemic reactions include chills, fever, malaise, apprehension, abdominal pain and diarrhea (Halstead,
1978). In more severe cases it can cause hemolysis, acute renal failure (Guess et al., 1982), breathing difficulty, convulsion, and arrhythmia (Russel, 1966).

Treatments for P. physalis venom intoxication are only supportive and palliative. No antisera or antidotes are known (Hessinger, 1988). Topical application of vinegar (4-6% acetic acid in water) to deactivate nematocysts and relieve pain in first aid has been practiced (Burnett et al., 1983). It is now questioned since recent research finds that it causes further discharge of the nematocysts (Exton, 1988). Manually debriding the area of adherent tentacles and immobilizing or resting the wound area for 30 to 60 minutes can reduce envenomation (Halstead, 1987). Indomethacin, and especially prednisone, relieve toxic responses. Analgesics and other agents can also be used according to the condition (Auerbach and Hays, 1987; and Burnett et al., 1983). Verapamil has been suggested for use against the cardiac toxicity of the venom (Burnett and Calton, 1987a).

Immunological and hematological effects

Most victims develop an IgG antibody, and many, an IgE antibody against the venom. Specific elevated IgG and IgE may persist for several years. Serologic cross-reactivity between Chrysaora quinquecirrha (sea nettle), P. physalis (Russo et al., 1983a) and other species of jelly fish (Burnett et al., 1988) occurs. It has been claimed that venoms from these organisms as well as sea anemones, sea wasps and rattlesnake have immunologically identical sites on their molecules (Russo et al., 1983b).

Hemolytic activity (Tamkun and Hessinger, 1981) and cytolysis of mast cells along with release of histamine (Flowers and Hessinger, 1981) have been reported. Glycophorin was a possible binding site for the hemolytic component of the venom (Lin and Hessinger, 1979). From the electron microscopic evidence Cormier (1984) suggested that the venom caused histamine release via a rapid, short-duration (the first 2 min.) exocytosis of granules and a slower, long-duration lysis of mast cells. The exocytosis required calcium ion and energy. In contrast Flowers and Hessinger (1981) showed that the cytolytic histamine release depended on
temperature but not calcium ion or energy. The extent of histamine release was maximized broadly over the rage of 10-30 °C.

Neuromuscular effects

*P. physalis* venom usually affects the nervous system before it affects voluntary muscles. The extract from *P. physalis* tentacles was reported to paralyze fiddle crabs (Welsh, 1956). When 16.2 µg of dried venom was injected into the ventral lymph sac of frogs, spinal reflexes and sciatic reflexes disappeared. Postural andrighting reflexes, that reflected the function of the central nervous system, deteriorated (Lane, 1960b; Larsen and Lane, 1970a). Intramuscular injection of adequate crude venom into fish was followed by disorientation and motor paralysis at the injection site (Lane, 1960b).

After intraperitoneal injection of the venom into the rat, immediate onset of toxic symptoms were observed including increased activity and tremors followed by ataxia, decreased muscle tone, flaccid paralysis, anoxic convulsions and death. The neuromuscular abnormalities were accompanied by slower and labored breathing, cyanosis, defecation, aphrodisia and marked myosis (Lane and Dodge, 1958).

*P. physalis* venom produces contraction in numerous skeletal and smooth muscle preparations (Larsen *et al*., 1987). The venom is suggested to interfere with the binding of calcium ion to the sarcoplasmic reticulum and its reuptake into this reservoir (Calton and Burnett, 1973b). The venom slowly contracts isolated ileum of guinea pigs (Burnett *et al*., 1975). The contraction is not altered by treatment with an antihistaminergic, an anticholinergic, or a ganglionic blocking agent. It is postulated that the venom depolarizes the cell membrane (Garriott and Lane, 1969).

Vascular effects

Vascular effects of *P. physalis* venom on animals are prominent. Rat arterial pressure was reported to be depressed in immediate response to *P. Physalis* venom followed by rising toward normal in 1 to 4 minutes (Garriott and Lane, 1969). The venom also prolonged the pressor effect of epinephrine by 2 to 3 times. Methysergide, an antiserotonin agent,
counteracted the increased permeability of rat cutaneous capillaries caused by the venom. Prior intravenous administration of piripost, a leukotriene inhibitor, has been shown to reduce the cutaneous vascular leakage induced by the venom (Burnett and Calton, 1986).

*P. physalis* venom consistently produced vasodilation in dog skeletal muscles *in vivo*. This was blocked by sodium meclofenamate, an inhibitor of cyclooxygenase, but not by cholinergic blockers such as atropine, or by adrenergic blockers such as phentolamine, or by histaminergic blockers such as cimetidine (Loredo et al., 1985). The venom produced relaxation in isolated rabbit arterial rings precontracted with norepinephrine. This action was also inhibited by sodium meclofenamate, but not by atropine, propranolol, or quinacrine (Loredo et al., 1986). It was suggested that *P. physalis* venom stimulated the synthesis of an endogenous vasodilatory ecosinoid.

It was showed that the vascular relaxation produced by *P. physalis* venom was endothelially dependent. After the endothelium was removed by gently rolling a 5-0 silk-threaded rabbit femoral artery ring over a moistened paper toweling, the ring no longer relaxed in the presence of the venom. This implied that the venom-induced relaxation of the vascular smooth muscle was mediated by the vascular endothelium (Luo et al., submitted).

**Cardiac effects**

In the literature only very limited research has been reported on the cardiac effects of *P. physalis* venom. Arrhythmia and conduction defects in mammalian heart were reported (Larsen et al., 1987). A dose of 300 µg per kg or larger reduced the heart rate of the rat (Garriott and Lane, 1969).

Electrocardiograms recorded from the rat showed that the venom affected the cardiac conduction system in proportion to the dosage administered. There was an increase in ventricular repolarization time followed by disruption of sinoatrial node activity and the development of atrioventricular block. A lower dose of *P. physalis* venom prolonged the Q-T interval suggesting that the venom interfered with the repolarization
process. At a higher dose an abnormally shortened P-R interval was observed and the P wave was from inverted to double. Shortened P-R intervals and inverted P waves suggested accelerated atrioventricular conduction and retrograde transmission of excitation waves over the atria, probably caused by a shift in the site of pacemaker activity from the sinoatrial node to a point near to the atrioventricular node. Double P waves indicated a second degree (2:1) atrioventricular block. Lethal doses caused ECG patterns in the rat similar to those of ventricular paroxysmal tachycardia or fibrillation. That the venom caused generalized depolarization of cell membranes by possible interference with Na+-K+ pump was suggested (Larsen and Lane, 1966).

In dogs, *P. physalis* venom at sub-lethal doses was found to increase cardiac output and systemic blood pressure, to raise serum potassium level, and to reduce serum sodium level. At higher doses, the venom caused arrhythmias similar to those of ouabain intoxication, such as P-R interval reduction, P wave suppression, A-V block, ectopic pacemaker activity and re-entry extrasystole. Infusion of KCl could reverse the arrhythmias (Hasting *et al.*, 1967).

A dose-dependent positive inotropic effect was observed in isolated rabbit atria (Bonlie *et al.*, 1988). The effect was directly related to the extracellular calcium concentration. Atenolol, a β receptor blocker, only partially inhibited the increased myocardial contractility caused by the venom. Dantrolene, an inhibitor of intracellular calcium release, was found to be ineffective in antagonizing the effect of the venom. Verapamil, a calcium channel blocker, was reported to be able to counteract the cardiac effect of the venom and delay death in mice (Burnett *et al.*, 1985).

**Hypotheses in the Mechanism of the Cardiovascular Actions of *P. physalis* Venom**

In recent years it has been suggested that *P. physalis* venom increases the transport of certain monovalent and divalent cations across biological membranes. The increased transport is facilitated by the
holes on the membrane (Burnett and Calton, 1987b). The finding that the venom increases permeability of frog skin to sodium ion without interfering with the active transport system directly, is so far one of few direct evidences to support this hypothesis (Larsen and Lane, 1970b). Nevertheless, it is not clear whether *P. physalis* venom creates a hole, or serves as an ionic channel itself. The size and the specificity of the hole are questionable.

Intravenous administration of verapamil has been reported to significantly delay death in mice that had been intravenously injected with one to three doses of LD50 *P. physalis* venom. Interfering with calcium channels by the venom is implicated (Burnett et al., 1985). However, ionic fluxes across the myocardial membrane under the influence of the venom have never been revealed in the literature.

Previous studies suggest that *P. physalis* venom relaxes vascular smooth muscles by stimulating vasodilatory ecosinoid synthesis by vascular endothelium (Loredo *et al.*, 1986; and Luo *et al.*, submitted). Endothelium-derived relaxing factor (EDRF) is known to play a major role in vascular smooth muscle relaxation (Cherry and Gillis, 1987; Furchgott, 1983; and Ignarro *et al.*, 1986). Nevertheless the relationship between *P. physalis* venom-produced vascular relaxation and EDRF has not been examined. Previous findings only involve the effect of the venom on the vascular endothelium. The direct effect of the venom on vascular smooth muscle has not yet been studied.

This work is to further investigate the mechanism of certain cardiovascular effects of *P. physalis* venom and to explore some of the questions posed by previously published work. I propose that in the heart, the venom produces a positive inotropic effect by either opening existing calcium channels of myocardial membranes, or by forming specific calcium channels. In addition to calcium, the venom may modify other ionic fluxes across the cell membrane either directly or indirectly. In the vasculature, *P. physalis* venom produces relaxation by increasing the synthesis of a vasodilatory ecosinoid by the vascular endothelium independently of endothelium-derived relaxing factor (EDRF). In addition to the
effect on the vascular endothelium the venom may also possess a direct
effect on vascular smooth muscles. Thirdly, *P. physalis* venom may or may
not create a non-specific hole in the cell membrane. The size of the hole can
be identified by the passage of certain molecules such as trypan blue and
glucose.
METHODS AND MATERIALS

Rabbit Femoral Arterial Rings

Preparation of rabbit femoral arterial rings

Femoral arteries were dissected and removed from male rabbits (2-3 kg) under anaesthesia with ketamine (50 mg/kg, i.m.) followed by sodium pentobarbital (25 mg/kg, i.v.). The unbranched arteries were placed in chilled, aerated (95% O₂, 5% CO₂) Krebs-Henseleit solution (containing 118 mM NaCl, 5.4 mM KCl, 12.5 mM NaHCO₃, 2.5 mM CaCl₂, 1.12 mM MgSO₄, 1.2 mM KH₂PO₄ and 10 mM D-glucose), cleaned of fat and connective tissues under the microscope, and cut into ring segments of 3 mm in length.

The arterial rings were mounted to a strain gauge and immersed in aerated (95% O₂, 5% CO₂) Krebs-Henseleit solution maintained at 37°C (Fig. 1). The base tension was gradually adjusted to 500 mg at a rate of 100 mg/5 minutes. The rings were allowed to equilibrate for 60 minutes. Between each sequence of addition of different agents the bath solution was changed twice, and the rings were allowed to equilibrate back to 500 mg base tension. Changes in tension over time were recorded by a servowriter (model 8373-20 recorder, Cole-Parmer Instrument Company, Chicago, ILL).

ED70 and P. physalis venom dose response curves

Norepinephrine (NE), phenylephrine (PE), clonidine, or KCl was used at a logarithmic dose scale until the maximal vascular contraction was obtained, and 70% of the maximal effective dose (ED70) was calculated from the individual dose response curves. Arterial rings were precontracted with norepinephrine ED70 (2 μM), phenylephrine ED70
Fig. 1 Krebs-Henseleit solution bath. Rabbit femoral arterial rings were mounted to strain gauges and immersed in aerated (95% O₂, 5% CO₂) Krebs-Henseleit solution bath (pH 7.4) at 37°C. The base tension was adjusted to 0.5 gram gradually over a period of 25 minutes (100 mg/5 minutes) and the ring was allowed to equilibrate for 1 hour. Between each sequence of addition of agents the bath was changed twice and the ring equilibrated back to 0.5 gram base tension.
(1 μM), clonidine ED70 (0.73 μM), KCl ED70 (33 mM), or PGF$_{2\alpha}$ (10-30 μM).

Prior to all experiments the presence of an intact vascular endothelium was verified by the dilatory response of NE or PE precontracted rings to methacholine (1 μM). *P. physalis* venom dose-response was derived by treating NE or PE precontracted, intact rings with increasing doses of *P. physalis* venom (2-400 μg/ml).

**Comparison of the relaxations caused by EDRF and *P. physalis* venom**

In the control group methacholine (1 μM) or *P. physalis* venom (3.3-6.6 μg/ml) was applied to NE precontracted, intact rings. In the treated group methylene blue (10 μM), a guanylate cyclase inhibitor, was added to NE precontracted, intact rings followed by methacholine (1 μM) or *P. physalis* venom (3.3-6.6 μg/ml).

**The direct effect of *P. physalis* venom on vascular smooth muscles**

KCl (33 mM) was used to depolarize vascular smooth muscles. In the control group, *P. physalis* venom (3.3-6.6 μg/ml) was applied to KCl depolarized rings with or without an intact endothelium (verified by the response to methacholine). In the treated group dibenzyline (1.5 μM) or yohimbine (1 μM) was added to KCl depolarized rings followed by the venom (3.3-6.6 μg/ml).

The actions of *P. physalis* venom on intact arterial rings with different tensions were tested. In the high tension group, rings were precontracted by NE followed by addition of the venom (3.3-6.6 μg/ml). In the low tension group the base tension was gradually lowered to and stabilized at 0.3 gram for 15 minutes, then the same amount of venom was added to the bath.
Rat Atrial Preparations

Male Sprague-Dawley rats (200-350 g) were decapitated. The heart was quickly removed and washed with chilled, aerated (95% O₂, 5% CO₂) Krebs-Henseleit solution twice. The atria including the SA node area were carefully dissected from the ventricles, mounted to the strain gauge, immersed in aerated (95% O₂, 5% CO₂), 37°C Krebs-Henseleit bath. The base tension was adjusted to 100 mg (Tarizzo and Rubio, 1982). The atria were equilibrated in the bath for 1 hour. Between each addition of different agents, the bath solution was changed three to four times and the atria were allowed to equilibrate back to 100 mg base tension.

Isoproterenol (1 nM-1 μM) and methacholine (0.1-10 μM) were used to test the responsiveness of the atrial preparation prior to experiments. P. physalis venom 0.005-1.5 μg/ml was added to the bath at a calcium concentration of 0.5 mM, 2.5 mM, 4.5 mM or 6.5 mM. The magnitude of developed tension and the rhythm of heart beat were recorded by a polygraph (Grass, model 7D, Grass Instrument Co. Quincy, MA).

Cultured Embryonic Chick Heart Cells

Embryonic chick heart cell culture

Fertile chicken eggs were incubated at 40°C for ten days. The cell culture procedure was a modification of a conventional method (Kim and Smith, 1986a, 1986b, 1987a and 1987b; Laurent et al., 1986; Kim et al., 1984 and 1987; Murphy et al., 1983; Murphy et al., 1988; Leatherman et al., 1987 and Masters et al., 1985). All steps of cell culture utilized aseptic techniques. The eggs were washed with 70% ethanol and cracked with sterile
forceps. The heart was removed from the chick embryo, washed with chilled Ca\(^{2+}\), Mg\(^{2+}\)-free Hank's solution (pH 7.4, containing 5.36 mM KCl, 0.44 mM KH\(_2\)PO\(_4\), 137 mM NaCl, 0.34 mM Na\(_2\)HPO\(_4\), and 5.55 mM glucose) twice, and minced into very fine tissue pieces.

Trypsin (0.05%, weight/volume) in Ca\(^{2+}\), Mg\(^{2+}\)-free Hank's solution was used to digest the minced heart tissue. The digestion process was carried on in an incubator at 37\(\degree\)C with shaking (60 times/min). In the conventional method, the heart tissue was digested for five times, 7-10 minutes each time. In this study it was observed that clumps of heart cells maintained spontaneous contraction in 0.05% trypsin (in Ca\(^{2+}\), Mg\(^{2+}\)-free Hank's solution) for more than 6 hours in room temperature. Embryonic chick heart cells which had been digested in 0.05% trypsin at 37\(\degree\)C for 3 hours still preserved their viability and formed a monolayer beating cell culture. According to these observations, the length of time for tissue digestion was increased. The modification increased cell harvest four to five fold. With the modified method the first digestion lasted 10 minutes. Then the supernatant fluid was discarded and the tissue was washed with chilled Ca\(^{2+}\)-Mg\(^{2+}\)-free Hank's solution once or twice to remove red blood cells, digested endothelial and epithelial cells. The second digestion lasted one hour while the third and the fourth 15 minutes each. Each time the supernatant from the digestion was collected and transferred to a conical tube. An equal volume of chilled medium containing 10% fetal calf serum (FCS), 40% medium 199, 49% balanced salt solution (consisting of 116 mM NaCl, 1.0 mM NaH\(_2\)PO\(_4\), 0.8 mM MgSO\(_4\), 1.18 mM KCl, 26.2 mM NaHCO\(_3\) and 5 mM glucose), and 1% penicillin-streptomycin-amphotericin B antibiotic-antimycotic solution (10,000 units-10 mg-25 mg per ml) was added
to the supernatant to discontinue the action of trypsin. The diluted supernatant was centrifuged at 600 rpm for 5 minutes. The cell pellet was resuspended in a culture medium containing 6% FCS, 40% medium 199, 53% balanced salt solution and 1% antibiotic-antimycotic solution (the final composition of the culture medium: 144 mM Na+, 4.0 mM K+, 0.97 mM Ca2+, 18 mM HCO3, 131 mM Cl-, with 100 unit penicillin, 0.1 mg streptomycin, 0.25 mg amphotericin B per ml culture medium). All suspensions were pooled into a 25 cm² or a 100 cm² tissue culture flask (according to the number of cells) and incubated at 37°C with a 5% CO₂ humidified atmosphere for one hour to facilitate differential adherence of the fibroblasts, and to increase the number of the heart cells in the suspension.

After one hour of incubation, a small drop of the cell suspension was taken to microscopically estimate cell number on a hemocytometer. According to the total number of cells, 6% FCS medium was used to make a diluted cell suspension with 2 x 10⁶ cells/ml (Fig. 2). Seven million cells together with 3.5 ml media were plated in one Falcon 60 x 15 mm tissue culture dish in which there were two glass cover slips (22 x 22 mm). The cells were incubated at 37°C in a 5% CO₂, humidified atmosphere. The medium was changed every other day. The experiment was performed on the third day when a monolayer of spontaneously beating heart cell culture was well formed.

45Ca²⁺ and ²²Na⁺ influxes

Cultured embryonic chick heart cells were preincubated in N-[2-hydroxyethyl] piperazine-N¹-[2-ethanesulfonic acid] (HEPES) buffered solution (HEPES 5 mM, KCl 4 mM, MgCl₂ 0.5 mM, CaCl₂ 0.9 mM, NaCl 140 mM, pH 7.35) at 37°C for 5-10 minutes (to allow the cell to adjust to the new
Fig. 2 Flow diagram of embryonic chick heart cell culture. All procedures utilized aseptic techniques. The length of time for digesting the heart tissue has been modified from the conventional method in which the tissue was only digested for 7-10 minutes for 5 times. The modification increased heart cells harvest 4-5 fold yet the viability of the cells was still preserved. The digestion solution contained 0.05% trypsin (weight/volume) in Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-free Hank's. 10\% FCS medium consists of 10\% fetal calf serum (FCS), 40\% medium 199, 49\% balanced salt solution, and 1\% penicillin-streptomycin-amphotericin B antibiotic-antimycotic solution. 6\% FCS medium consists of 6\% FCS, 40\% medium 199, 53\% balanced salt solution, and 1\% penicillin-streptomycin-amphotericin B antibiotic-antimycotic solution.
EMBRYONIC CHICK HEART CELL CULTURE

fertile eggs incubated at 40°C for 10 days

aseptically remove the heart

wash with Ca-Mg-free Hank's x 2 then mince into small pieces

0.05% trypsin digestion

1 st. for 10 min.

discard the supernatant

w Ashley with chilled Ca-Mg-free Hank's sol.

2 nd. for 60 min.

add equal vol. of chilled 10% FCS med.

3 rd and 4 th for 15 min. each

add equal vol. of chilled 10% FCS med.

1 st. for 10 min.

discard the supernatant

wash with chilled Ca-Mg-free Hank's sol.

2 nd. for 60 min.

add equal vol. of chilled 10% FCS med.

3 rd and 4 th for 15 min. each

add equal vol. of chilled 10% FCS med.

600 rpm for 5 min.

resuspended with 6% FCS med.

pool all cells together
incubated at 37°C for 1 hr.
(differential adherence)

count and plate
environment). In the calcium channel blocker (diltiazem or verapamil) or sodium channel blocker (flecainide) treated groups, the cultured heart cells were preincubated with the blocker (10^-7 to 10^-1 M diltiazem; 10^-7 to 10^-2 M verapamil; or 10^-7 to 10^-3 M flecainide) in HEPES buffered solution at 37°C for 20 minutes. Cultured embryonic chick heart cells on cover slips were transferred to the cover slip holder, then incubated in a chamber containing 45Ca^2+ (5 μCi/ml) or 22Na+ (1 μCi/ml) HEPES buffered solution with or without *P. physalis* venom (0.2 μg/ml) at 37°C for 0, 10, 20, 30, 60, 120, 300 seconds respectively (Fig. 3). To determine the *P. physalis* venom dose response relationship the cultured heart cells were incubated in 45Ca^2+ or 22Na+ HEPES buffered solution at 37°C for 1 minute with 0.02, 0.06, 0.2, 0.6, 2, 6 μg/ml venom respectively. Immediately after the incubation, each cover slip was washed sequentially in two 80 ml volumes of chilled (3 to 4 °C) HEPES buffered solution (pH 7.35), 8 seconds in each, with stirring. NaOH (1.5 ml, 1 N) was used to digest the cultured heart cells on two cover slips (in the lid of a 60 x 15 mm tissue culture dish) for 10 minutes. Two aliquots of 0.5 ml digestion solution were each mixed with two 5 ml volumes of scintillation cocktail for counting radioactivity and two aliquots of 50 μl digestion solution were each placed into two test tubes for Bradford protein assay (Kim and Smith, 1986a and 1987a; Leatherman *et al.*, 1987; Martinez *et al.*, 1989; Kimelberg *et al.*, 1989 and Garty *et al.*, 1989).

**2-Deoxy-D-[2-3H] glucose uptake and 86Rb+ influx**

Cultured embryonic chick heart cells were preincubated in 37°C HEPES buffered solution for 5-10 minutes. The cells were then incubated in HEPES buffered solution containing 2-deoxy-D-[2-3H] glucose (2 μCi/ml) or 86Rb+ (2 μCi/ml) at 37°C, with or without *P. physalis* venom (0.2 μg/ml), for
Fig. 3 The glass cover slip holder and the radioactive isotope solution chamber. The chamber which contained HEPES buffered solution (pH 7.35) with a radioactive isotope was maintained at 37 °C in a water bath. Cultured embryonic chick heart cells on cover slips were transferred to the cover slip holder and incubated in the chamber for the described amount of time. Immediately after the incubation each cover slip was sequentially washed in two 80 ml volumes of 3-4 °C HEPES buffered solution, 8 seconds in each, with stirring. NaOH (1.5 ml, 1 N) was used to digest the heart cells after washing.
1, 2, 5, 15, 30 minutes respectively (Gordon et al., 1986; Segal et al., 1977; Frelin et al., 1986; Otero and Szabo, 1988; Vornanen, 1984). To determine the dose response relationship, cultured heart cells were incubated in HEPES buffered solution containing 2-deoxy-D-[2-3H] glucose at 37 °C for 15 minutes with P. physalis venom 0.02, 0.2, 2, or 20 µg/ml, or in HEPES buffered solution containing 86Rb+ for 5 minute with P. physalis venom 0.02, 0.06, 0.2, 0.6, 2, or 6 µg/ml.

86Rb+ efflux

Cultured embryonic chick heart cells were incubated in HEPES (10 mM) buffered medium with 86Rb+ (2 µCi/ml) at 37°C for 2 hours to allow the equilibration of intra- and extracellular 86Rb+. The cells were then incubated in 86Rb+ free, HEPES buffered medium at 37°C with or without P. physalis venom (2 µg/ml) for 0, 10, 20, 30, 60, 120, 300 seconds respectively (Kino et al., 1985; Hunter and Nathanson, 1985). To determine the dose response relationship, 86Rb+ loaded cultured heart cells were incubated in 86Rb+ free, HEPES buffered media for one minute with P. physalis venom 0.02, 0.06, 0.2, 0.6, 2, 6, or 20 µg/ml.

Microscopic observation of contractility and rhythm

Embryonic chick heart cells were plated in a 24 well plate in a density of 5.66 x 10^5 cells/cm^2. On the third day the medium was changed to one containing only 3% fetal calf serum and buffered with 10 mM HEPES. The observation was made in 37°C room air under a microscope equipped with video camera and TV screen. P. physalis venom was added according to an increasing dose scale (0.02, 0.06, 0.2, 0.6, 2, 6 µg/ml). Rhythm of the heart cell was counted during the first two minutes and the contraction state was recorded by VCR.
Trypan blue test

Trypan blue 20 μl/ml was applied to the cultured heart cells followed by the addition of *P. physalis* venom from 0.02 μg to 20 μg/ml. Intracellular color change was closely observed under the microscope immediately, 1 hour, 4 hours and 24 hours after the treatment. The cultured heart cells were incubated in a 37 °C, humidified 5% CO₂ atmosphere between observations.

Cell density correction

Twenty-four hours before an ionic flux experiment, cultured heart cells were incubated in a medium containing L-[4, 5,-³H] leucine (0.1 μCi/ml). The amount of incorporated radioactive leucine was consistent with the amount of total cell protein (Kim and Smith, 1986a and 1987a). Samples from all experiments were counted for both ³H and individual ⁴⁵Ca²⁺, ²²Na⁺, or ⁸⁶Rb⁺ by a Beckman liquid scintillation β counter (model LS 5801, Beckman Instruments, Fullerton, CA). Counting of ³H allowed for correct estimation of the amount of protein in each sample. The calculation included three steps. First, to estimate the leaking of an individual channel with higher energy level (e.g. ⁴⁵Ca²⁺, ²²Na⁺, or ⁸⁶Rb⁺) to ³H channel (one of lower energy), a pure ⁴⁵Ca²⁺, ²²Na⁺, or ⁸⁶Rb⁺ sample was counted in both its own and ³H channel. The percentage of leaking of an individual channel to ³H channel was calculated by dividing the count per minute (cpm) from ³H channel by the cpm from the individual channel. Pure ³H counts from L-[4, 5,-³H] leucine in each sample having two isotopes was obtained after the portion of leaking from the other isotope was deducted (pure ³H cpm from L-[4, 5,-³H] leucine = cpm [³H channel] - cpm [other channel] x % leaking). Secondly, to derive the cpm (L-[4, 5,-³H] leucine)/mg protein ratio of an
experiment, about 10-20% of the samples of an experiment were randomly selected for the Bradford protein assay (Bradford, 1976). The ratio of cpm (L-\([4, 5, ^{-3}H]\) leucine)/mg protein of a sample was calculated by dividing cpm (L-\([4, 5, ^{-3}H]\) leucine) by mg protein for the sample. When all the results for the selected samples were averaged, the cpm (L-\([4, 5, ^{-3}H]\) leucine)/mg protein ratio of one particular experiment was derived. Thirdly, the amount of protein for each sample of an experiment was calculated by dividing cpm (L-\([4, 5, ^{-3}H]\) leucine) by the cpm (L-\([4, 5, ^{-3}H]\)) leucine per mg protein ratio of that experiment. For 2-deoxy-D-\([2^{-3}H]\) glucose uptake experiment, two 50 \(\mu\)l aliquots of each sample were taken for protein estimation by Bradford protein assay.

*Calculation of moles of a specific ion or molecule*

To calculate the moles of a specific ion the following steps were taken: (1) calculation of disintegrations per minute (dpm) using the formula, dpm = (cpm-background)/efficiency; (2) conversion of dpm to \(\mu\)Ci using the formula, \(1 \mu\)Ci = 2.22 \(\times\) 10\(^6\) dpm; (3) correction factor for decay using the formula, \(X_o = X(2)\frac{t}{T}\) (where: \(T\) = half life of the isotope, \(t\) = the time elapsed from the reference day, \(X_o\) = the corrected value of activity, and \(X\) = the uncorrected value of activity); (4) calculation of moles of an ion or molecule using the specific activity of each ion or molecule, gram/mCi or mole/mCi; (5) correction by a dilution factor according to the portion for scintillation counting from the whole sample.

*Bradford protein assay*

The microprotein assay was used (Bradford, 1976). This method was reported to be very reproducible and rapid with the dye binding process. The dye binding assay was approximately four times more sensitive than
the Lowry method (Bradford, 1976; Lowry et al., 1951). The standard protein samples included 0, 1.44, 2.88, 5.76, 11.52, 23.04 μg protein respectively in 0.8 ml distilled water and 0.2 ml BIO-RAD protein dye (total 1 ml). The standards were well mixed and placed at room temperature for 5 minutes. Optical density (O.D.) at wavelength 595 micron was measured by the use of a spectrophotometer (Stasar II, Gilford Instruments Laboratories INC., Oberlin, OH). A regression line was derived by plotting the O.D. values against the μg protein values.

To quantify amount of protein in each sample, 0.75 ml of distilled water and 0.2 ml dye were added to 50 μl of the sample protein solution (total 1 ml), well mixed and placed at room temperature for 5 minutes before the measurement of O.D. The amount of protein (in 50 μl) was calculated by the regression line derived from the standard samples. The total amount of protein of a sample was calculated by multiplying the amount of protein in 50 μl by a dilution factor (according to the proportion of 50 μl aliquot in the total sample).

Preparation of *P. physalis* Venom

Portuguese man-of-war were collected on the shore near Miami, FL. The tentacles were frozen at -20°C within a few hours after the collection. Nematocysts were extracted by breaking up the tentacles mechanically followed by filtration through double layers of window screen (1.6 x 1.6 mm). The filtrate was centrifuged at 350 rpm, 4°C for 5 minutes. The pellet was resuspended in 12 ml of 0.2 M NaCl. The volume was doubled with more KCl solution every 10 minutes until 200 ml was reached. Then the suspension was again centrifuged at 350 rpm, 4°C for 5 minutes. The above
procedure was repeated with 0.4 M, 0.7 M and 1.0 M NaCl respectively. The resulting pellet of nematocysts was homogenized in 150 mM NaCl plus 25 mM Tris-acetate (pH 6) in order to discharge the nematocysts. Discharged nematocysts were removed by centrifugation at 15,000 rpm, 4°C for 15 minutes. The supernatant fluid (venom) was collected and stored at -20°C (Tamkun and Hessinger, 1981). The protein content of the venom was determined by Bradford protein assay.

**Statistical Analysis**

One way and two way analyses of variance, simple regression, correlation coefficient (r) and determination of coefficient (r²) were used in statistical analyses. All values represent the mean ± standard error of the mean (S.E.)

**Materials Used**

Norepinephrine, phenylephrine, methacholine, methylene blue, clonidine, dibenzyline, yohimbine, KCl, isoproterenol, diltiazem, verapamil, Ca²⁺, Mg²⁺-free Hank’s salt, trypan blue, penicillin-streptomycin-amphotericin B antibiotic-antimycotic solution, trypsin, and HEPES were purchased from Sigma Chemical Company (St. Louis, MO). Medium 199 and fetal calf serum were purchased from Irvine Scientific Company (Irvine, CA). Fertile eggs were purchased from Hy-line Corporation (Lakeview, CA). ⁴⁵Ca²⁺ and 2-deoxy-D-[²-³H] glucose were purchased from ICN Biochemical Incorporation (Costa Mesa, CA). ⁸⁶Rb⁺ was purchased from E. I. Dupont de Nemour & Co. Incorporation (Wilmington, DE). ²²Na⁺ and L-[⁴, ⁵-³H] leucine were purchased from Amersham
Corporation (Arlington Heights, ILL.). Buget solve, a scintillation cocktail, was purchased from Research Product International Corporation (Mt. Prospect, ILL). The protein assay package was purchased from BIO-RAD Laboratories (Richmond, CA). Glass cover slips purchased from Fisher Scientific/Instrumental Laboratories (Pittsburgh, PA) were washed with 1 N HCl, distilled water, followed by 95% ethanol before autoclave sterilization. Falcon tissue culture dishes were purchased from VWR Scientific Incorporation (San Diego, CA). PGF$_2\alpha$ and flecainide were donated by Upjohn Company (Kalamazoo, MI). All the $P.\ physalis$ venom used was obtained from the same purified batch.
RESULTS

Rabbit femoral arterial rings

*P. physalis venom dose response*

Norepinephrine or phenylephrine precontracted (9.01 ± 0.465 g and 9.35 ± 0.640 g respectively), intact rabbit femoral arterial rings were significantly relaxed by *P. physalis* venom in a dose-dependent manner (p < 0.05, Fig. 4).

**Comparison of vascular relaxation induced by EDRF and *P. physalis* venom**

Both methacholine and *P. physalis* venom significantly relaxed norepinephrine precontracted, intact rings (-1.88 ± 0.279 g and -0.8 ± 0.199 g respectively, p < 0.05, Fig. 5). Relaxation caused by methacholine lasted less than 60 seconds (Fig. 6a), but relaxation produced by the venom persisted more than 30 minutes (Fig. 6b). Methylene blue completely blocked the relaxation caused by methacholine but had no effect on the relaxation produced by the venom (Fig. 5). In fact, venom-induced vascular relaxation was slightly enhanced in the presence of methylene blue (-1.1569 ± 0.523 g, Fig. 5). Methacholine appeared to induced a mild vascular constriction when methylene blue was present (p = 0.05).

**The direct effect of *P. physalis* venom on vascular smooth muscle**

*P. physalis* venom significantly produced vascular contraction in KCl depolarized rings (6.918 ± 0.322 g) with or without an intact endothelium (1.94 ± 0.229 g and 1.18 ± 0.364 g respectively, p < 0.05, Fig. 7). The vascular contraction produced by the venom was completely abolished by 1.5 μM dibenzyline and by 1 μM yohimbine (Fig. 7).

The venom also produced significant vascular constriction in PGF$_{2\alpha}$
Fig. 4 *P. physalis* venom dose responses of norepinephrine (---) and phenylephrine (-----) precontracted rabbit femoral arterial rings. Endothelium intact rings were precontracted with norepinephrine (2 μM) or phenylephrine (1 μM). *P. physalis* venom was added to the bath in an increasing dose manner (2-400 μg/ml). Each point represents the mean ± S.E. The relaxation produced by the venom is significant (p < 0.05).
Fig. 5 Vascular relaxation produced by endothelium-derived relaxing factor (EDRF) and by *P. physalis* venom. In control groups (-MB) intact rabbit femoral arterial rings were precontracted with norepinephrine (2μM), followed by the addition of methacholine (1 μM) or *P. physalis* venom (3.3-6.6 μg/ml). In methylene blue treated groups (+MB), methylene blue (10 μM) was applied to norepinephrine precontracted, intact rings, followed by the addition of methacholine (1 μM) or the venom (3.3-6.6 μg/ml). Each bar and vertical line represent the mean ± S.E. Relaxations produced by methacholine and the venom are significant in the control group, but only the venom relaxed the methylene blue treated group significantly (p < 0.05).
Fig. 6 EDRF and *P. physalis* venom produced vascular relaxation recorded by a servowriter (redrawn). Tension change over time was recorded. a. Methacholine (MACH, 1µM) was applied to a norepinephrine (NE, 2 µM) precontracted, intact ring with or without methylene blue (MB). b. *P. physalis* venom (Ve, 3.3-6.6 µg/ml) was applied to a norepinephrine precontracted, intact ring with or without methylene blue. The broken line indicates a prolonged length of time.
Fig. 7 The direct effect of *P. physalis* venom on vascular smooth muscles. Endothelium-intact rings (endo. pre.) and endothelium-deprived rings (endo. depr.) were depolarized with KCl (33 mM) followed by the addition of *P. physalis* venom (3-3-6.6 µg/ml). In other two groups of KCl depolarized, intact rings, dibenzyline (1.5 µM) or yohimbine (1 µM) was applied before the same amount of the venom. Each bar and vertical line represent the mean ± S.E. Contractions induced by the venom in both endothelium-intact and -deprived groups are significant (*p* < 0.05).
precontracted (1.062 ± 0.212 g), intact rings and in rings with 0.3 gram tension (2.263 ± 0.475 g, 1.883 ± 0.857 g, respectively, p < 0.05, Fig. 8).

In summary, *P. physalis* venom produced vascular relaxation in adrenergic agonists (eg. norepinephrine, phenylephrine, and clonidine) precontracted, intact rings (Fig. 8). However, the venom caused vascular constriction in KCl and PGF$_{2\alpha}$ precontracted, intact rings and rings with 0.3 gram tension (Fig. 8). When the percentage tension change was compared for different agents, the venom caused the largest tension change in 0.3 gram base tension group (Fig. 9).

**Rat Atrial Preparation**

*P. physalis* venom had a positive inotropic effect on rat atrial preparation. This action was enhanced by increased extracellular calcium concentration (p < 0.05, Fig. 10). At low extracellular calcium level (0.5 mM), the effect of the venom was not evident.

At higher venom concentrations (1.5 μg/ml or higher), the rhythm was increased. Arrhythmia became progressively worse as the venom concentration increased. At the final stage, fibrillation and cessation of contraction were observed.

**Cultured Embryonic Chick Heart Cells**

*Calcium influx*

*P. physalis* venom significantly increased calcium influx of cultured monolayer beating embryonic chick heart cells (p < 0.01, Fig. 11). The elevation of calcium influx had a dose-dependent relationship (Fig. 12). The
Fig. 8 The effect of *P. physalis* venom on intact rabbit femoral arterial rings precontracted with different vasoactive agents or in lower tension. Endothelium-intact rabbit femoral arterial rings were precontracted with nor-epinephrine (NE, 2 μM), phenylephrine (PE, 1 μM), clonidine (0.73 μM), KCl (0.033 M), PGF₂α (10-30 μM), or stabilized at 0.3 gram tension (0.3 g ten.) followed by the addition of *P. physalis* venom (3.3-6.6 μg/ml). Each bar and vertical line represent the mean ± S.E. All bars are significantly different from the control (the precontracted tension, p < 0.05).
Fig. 9 Percentage of tension change caused by *P. physalis* venom in intact rabbit femoral arterial rings precontracted with different vasoactive agents or in lower tension. The % tension change was calculated by dividing the tension change (±gram) caused by *P. physalis* venom by the precontracted tension caused by norepinephrine (NE), phenylephrine (PE), clonidine, KCl, or PGF2α. In 0.3 gram tension group (0.3 g ten.), percentage of tension change was derived by dividing the tension change caused by the venom by 0.3 gram. The results then time 100. Each bar and vertical line represent the mean ± S.E.
Fig. 10 *P. physalis* venom dose response of rat atrial preparations with different extracellular calcium concentrations. The rat atrial preparation was mounted to a strain gauge and equilibrated in an aerated (95% O₂, 5% CO₂) Krebs-Henseleit bath at 37°C with 100 mg base tension. *P. physalis* venom (0.005-1.5 μg/ml) was added to the bath in an increasing manner with different calcium concentrations. Each point represents the mean ± S.E. The four lines are significantly different (p < 0.05). The developed tension at different concentrations of the venom is significantly different (p < 0.05).
Fig. 11 The effect of *P. physalis* venom on the calcium influx of cultured embryonic chick heart cells. Cultured embryonic chick heart cells were preincubated in HEPES buffered solution (pH 7.35) at 37°C for 5-10 minutes, then incubated in HEPES buffered solution containing $^{45}$Ca$^{2+}$ (5 μCi/ml) with or without the venom. Each point represents the mean ± S.E. The two lines are significantly different (p < 0.01).
**Fig. 12** *P. physalis* venom dose response of the calcium influx in cultured embryonic chick heart cells. Cultured embryonic chick heart cells were preincubated in HEPES buffered solution (pH 7.35) at 37°C for 5-10 minutes, then incubated in HEPES buffered solution containing $^{45}$Ca$^{2+}$ (5 μCi/ml) for one minute. *P. physalis* venom was added to the solution immediately before the incubation of the heart cells. Each point represents the mean ± S.E.
magnitude of response was more prominent when the time of exposure to the venom was longer.

The increased calcium influx was blocked by diltiazem (p < 0.01, Fig. 13) and verapamil (p < 0.01, Fig. 14). The blocking effects of these two calcium channel blockers were dose-dependent (Fig. 15 and Fig. 16). 0.1 M diltiazem or 0.01 M verapamil completely inhibited the effect of the venom at a level of 0.2 μg/ml (Fig. 13 and Fig. 14). Neither diltiazem nor verapamil blocked the calcium influx of the control group to a significant degree (p > 0.05). Diltiazem, a calcium channel blocker acting more specifically on the heart, appeared to be slightly more potent than verapamil in blocking the increased calcium influx caused by the venom (p < 0.01).

Sodium influx

*P. physalis* venom significantly increased the influx of sodium of culture embryonic chick heart cells (p < 0.01, Fig. 17). This effect was dose-dependent (Fig. 18). The increased sodium influx (picomole/mg protein) was a thousand times less than the increased calcium influx (nanomole/mg protein). The maximal increase for sodium influx was 122.24 ± 25.71% per minute more than the control while that for calcium influx was 627.48 ± 56.72% more (Fig. 19). The increased sodium influx was not inhibited by flecainide (0.001 M), a sodium channel blocker (p > 0.05), or by verapamil (0.01 M), a calcium channel blocker. Although the values of the group treated with the venom and verapamil were slightly lower than those treated with the venom alone, it was not statistically significant (p > 0.05, Table 1).

Rubidium influx

Rubidium influx of cultured embryonic chick heart cells was not
Fig. 13 The inhibitory effect of diltiazem on the increased calcium influx of cultured embryonic chick heart cells caused by *P. physalis* venom. In diltiazem (dil.) treated groups, cultured embryonic chick heart cells were preincubated in 0.1 M diltiazem in HEPES buffered solution (pH 7.35) at 37°C for 20 minutes while other groups were preincubated in HEPES buffered solution at 37°C for 5-10 minutes. Then the heart cells were incubated in HEPES buffered solution (pH 7.35) containing 45Ca2+ (5 µCi/ml) with or without the venom. Each point represents the mean ± S.E. Diltiazem completely inhibits the increased calcium influx (p < 0.01).
**Fig. 14** The inhibitory effect of verapamil on the increased calcium influx of cultured embryonic chick heart cells caused by *P. physalis* venom. In verapamil (vera.) treated groups, cultured embryonic chick heart cells were preincubated with 10^{-2} M verapamil in HEPES buffered solution (pH 7.35) at 37°C for 20 minutes while other groups were preincubated in HEPES buffered solution at 37°C for 5-10 minutes. Then the heart cells were incubated in 45Ca^{2+} (5 \mu Ci/ml) HEPES buffered solution with or without the venom. Each point represents the mean ± S.E. The increased calcium influx caused by the venom is complete inhibited (p < 0.01).
Fig. 15 Diltiazem dose response of the increased calcium influx caused by *P. physalis* venom. Cultured embryonic chick heart cells were preincubated with different doses of diltiazem (10^{-6} to 10^{-1} M) in HEPES buffered solution (pH 7.35) at 37°C for 20 minutes then incubated in HEPES buffered solution containing 45Ca^{2+} (5 μCi/ml) with or without the venom for one minute. Each point represents the mean ± S.E. The two lines are significantly different (p < 0.01).
Fig. 16 Verapamil dose response of the increased calcium influx in cultured embryonic chick heart cells caused by *P. physalis* venom. Cultured embryonic chick heart cells were preincubated with different doses of verapamil (10\(^{-7}\) to 10\(^{-2}\) M) in HEPES buffered solution (pH 7.35) at 37\(^\circ\)C for 20 minutes then incubated in HEPES buffered solution containing \(^{45}\)Ca\(^{2+}\) (5 \(\mu\)Ci/ml) with or without the venom for one minute. Each point represents the mean ± S.E. The two lines are significantly different (p < 0.01).
Fig. 17 The effect of *P. physalis* venom on the sodium influx of cultured embryonic chick heart cells. Cultured embryonic chick heart cells were preincubated in HEPES buffered solution (pH 7.35) at 37°C for 5-10 minutes, then incubated in HEPES buffered solution containing $^{22}\text{Na}^+$ (1 μCi/ml) with or without the venom. Each point represents the mean ± S.E. The two lines are significantly different (p < 0.01).
Fig. 18 *P. physalis* venom dose response of the sodium influx in cultured embryonic chick heart cells. Cultured embryonic chick heart cells were preincubated in HEPES buffered solution (pH 7.35) at 37°C for 5-10 minutes, then incubated in $^{22}\text{Na}^+$ (1 μCi/ml) HEPES buffered solution for one minute. *P. physalis* venom was added to the solution immediately before the incubation. Each point represents the mean ± S.E.
Fig. 19  Comparison of the increased calcium and sodium influxes of cultured embryonic chick heart cells under the influence of *P. physalis* venom. The magnitudes of the increased sodium influx and the increased calcium influx of cultured embryonic chick heart cells were compared in terms of % increase per minute ([treated - control] x 100/control) at 0.02, 0.06, 0.2, 0.6, or 2 µg/ml concentration of the venom. Each point represents the mean ± S.E.
altered by *P. physalis* venom at lower concentration such as 0.2 μg/ml (p > 0.05, Fig. 20). At a higher concentration (6 μg/ml), the venom significantly decreased the influx of rubidium (p < 0.01, Fig. 21).

**Rubidium efflux**

Rubidium efflux was significantly increased under the influence of *P. physalis* venom (0.2 μg/ml, p < 0.01, Fig. 22). The increased efflux was dose-dependent (Fig. 23). There was an exponential correlation between the calcium influx and rubidium efflux at the same venom level (0.02, 0.06, 0.2, 0.6, 2 μg/ml respectively, r = 0.992, r²=0.985, Fig. 24).

**2-deoxy-D-glucose uptake**

*P. physalis* venom had no effect on 2-deoxy-D-[2-3H] glucose uptake by embryonic chick heart cells (p > 0.05, Fig. 25). Only at a very high concentration (20μg/ml) did the venom significantly decrease the uptake of 2-deoxy-D-glucose into the cells (p < 0.01, Fig. 26).

**Microscopic observation**

With gradually increasing doses of *P. physalis* venom (from 0.02-2 μg/ml), cultured embryonic chick heart cells appeared to contract more completely. This seemed to be consistent with increased calcium influx caused by the venom. At high venom concentrations (more than 2 μg/ml) arrhythmia such as tachycardia, bradycardia, flutter, fibrillation occurred. Complete cessation of contraction of the heart cell usually followed brief fibrillation. When the venom concentration reached to 10 μg/ml or higher, all heart cells ceased to beat.

**Trypan blue test**

No intracellular staining was observed even in the presence of extremely high concentrations of *P. physalis* venom (20 μg/ml). When the
Fig. 20 The effect of *P. physalis* venom on the rubidium influx of cultured embryonic chick heart cells. Cultured embryonic chick heart cells were preincubated in HEPES buffered solution (pH 7.35) at 37°C for 5-10 minutes, then incubated in $^{86}$Rb+ (2 μCi/ml) HEPES buffered solution with or without the venom. Each point represents the mean ± S.E. The two lines are not different (p > 0.05).
Fig. 21 *P. physalis* venom dose response of the rubidium influx in cultured embryonic chick heart cells. Cultured embryonic chick heart cells were preincubated in HEPES buffered solution (pH 7.35) at 37 °C for 5-10 minutes, then incubated in HEPES buffered solution containing $^{86}$Rb$^+$ (2 μCi/ml) for 5 minutes with different dose of the venom (0.02-6 μg/ml). Each point represents the mean ± S.E. The rubidium influx at the point of 6 μg/ml venom is significantly different from the others (p < 0.01).
Fig. 22 The effect of *P. physalis* venom on the rubidium efflux of cultured embryonic chick heart cells. Cultured embryonic chick heart cells were incubated in HEPES (10 mM) buffered medium containing $^{86}\text{Rb}^+$ (2 μCi/ml) at 37°C for two hours, then incubated in $^{86}\text{Rb}^+$-free, HEPES buffered medium with or without the venom. Each point represents the mean ± S.E. The two lines are significantly different ($p < 0.01$).
Fig. 23 *P. physalis* venom dose response of the rubidium efflux in cultured embryonic chick heart cells. Cultured embryonic chick heart cells were incubated in HEPES (10 mM) buffered medium containing ^86^Rb+ (2 μCi/ml) at 37°C for 2 hours, then incubated in ^86^Rb+-free, HEPES buffered medium at for one minute with 0.02-6 μg/ml venom. Each point represents the mean ± S.E.
Fig. 24 The relationship between calcium influx and rubidium efflux of cultured embryonic chick heart cells. Calcium influx and rubidium efflux of cultured embryonic chick heart cells in the same P. physalis venom concentration (0.02, 0.06, 0.2, 0.6, 2 μg/ml for 5 points from left to right) were plotted as scatter graph with calcium influx on the abscissa and rubidium efflux (flowed out of the cell/mg protein content) on the ordinate. A line, $y = 0.34913 \times 0.42956^x$, that is closest to all the points is found ($r = 0.992, r^2 = 0.985$).
Fig. 25 The effect of *P. physalis* venom on 2-deoxy-D-[2-³H] glucose uptake by embryonic chick heart cells. Cultured embryonic chick heart cells were preincubated in HEPES buffered solution (pH 7.35) at 37°C for 5-10 minutes, then incubated in HEPES buffered solution containing 2-deoxy-D-[2-³H] glucose (2 µCi/ml) with or without the venom. Each point represents the mean ± S.E. The two lines are not different (p > 0.05).
Fig. 26 *P. physalis* venom dose response of 2-deoxy-D-[2-3H] glucose uptake by cultured embryonic chick heart cells. Cultured embryonic chick heart cells were preincubated in HEPES buffered solution (pH 7.35) at 37°C for 5-10 minutes, then incubated in HEPES buffered solution containing 2-deoxy-D-[2-3H] glucose (2 μCi/ml) for 15 minutes with *P. physalis* venom 0.02-20 μg/ml. Each point represents the mean ± S.E. The uptake of the glucose at 20 μg/ml venom level is significantly different (p < 0.01).
concentration reached to 6 μg/ml or higher, some cells started to detach from the culture dish. At 20 μg/ml level, about 20-30% of the cells detached. Intracellular staining by trypan blue in a small number of dead cells was observed only 24 hours after incubation with 20 μg/ml venom.
DISCUSSION

Rabbit Femoral Arterial rings

Indirect effects of *P. physalis* venom on vascular smooth muscles

In previous studies, *P. physalis* venom produced vasodilation in dog skeletal muscles *in vivo* (Loredo et al., 1985) and relaxation in norepinephrine precontracted rabbit arterial rings *in vitro* (Loredo et al., 1986). This vascular relaxation was dependent upon an intact endothelium. When the endothelium was removed, the venom no longer relaxed the ring (Luo et al., submitted). The relaxation was not attenuated by adrenergic, cholinergic or histaminergic antagonists but sodium meclofenamate, a cyclooxygenase inhibitor, was found to block the effect of the venom (Loredo et al., 1986). It was suggested that the venom stimulated vasodilatory prostaglandin synthesis in vascular endothelium.

Recently, an endothelium-derived relaxing factor (EDRF) has been reported (Cherry and Gillis, 1987; Furchgott, 1983; Ignarro et al., 1986; Martin et al., 1985; Rapoport and Murad, 1983; Rapoport et al., 1984; Rubanyi et al., 1987). EDRF diffuses from the vascular endothelium into the vascular smooth muscle where it stimulates guanylate cyclase activity. Increased intracellular cyclic GMP leads to smooth muscle relaxation probably by enhancing sequestration of calcium by the sarcoplasmic reticulum (Twort and Van Breemen, 1988). Acetylcholine causes vascular relaxation by stimulating EDRF release from the endothelium and acetylcholine-induced vascular relaxation has been used to indicate the presence of an intact vascular endothelium (Cherry and Gillis, 1987; Diamond and Chu, 1983; Halzmann, 1982; Ignarro et al., 1984; and Rubanyi et al., 1987).
Since *P. physalis* venom-induced vascular relaxation is endothelium-dependent, EDRF may be implicated as a possible mediator in venom-produced vascular relaxation. To test this possibility methacholine, an analogue of acetylcholine, and methylene blue, an inhibitor of guanylate cyclase are used. When the effect of EDRF is blocked by methylene blue, methacholine causes net vasoconstriction, in part by acting as an agonist for muscarinic receptors on smooth muscle cells, or perhaps also by stimulating the release of an endothelium-derived contracting factor, a 3000 dalton vasoconstrictive peptide (Harrison *et al.*, 1987; O'Brien *et al.*, 1987; and Shimodawa *et al.*, 1987).

In contrast to its effects with methacholine, methylene blue has no effect on the relaxation produced by the venom. The mechanism of the vasodilatory action of the venom, therefore, must not depend on EDRF. These findings further confirm the hypothesis that the venom stimulates the synthesis of a vasodilatory eicosinoid by the vascular endothelium.

In addition to cyclic GMP, cyclic AMP is crucial in vascular relaxation. The contraction of vascular smooth muscle cells is caused by increased levels of intracellular calcium. Calcium ions bind to calmodulin to form calcium-calmodulin (CaM) which then bind to myosin light chain kinase (MLCK), becoming an active enzyme complex (CaM-MLCK). CaM-MLCK phosphorylates the myosin light chain so that it can be activated by actin to elicit smooth muscle contraction. Antagonizing the effects of elevated intracellular calcium, intracellular cyclic AMP stimulates cyclic AMP-dependent protein kinase that phosphorylates the myosin light chain at a different site, deactivating it and, thereby, relaxing vascular smooth muscles (Adelstein and Sellers, 1987; and Stull *et al.*, 1988).
The literature reveals that arachidonic acid of endothelial origin stimulates the synthesis of both cyclic AMP and cyclic GMP in the arterial vascular smooth muscle and causes relaxation. Indomethacin, an inhibitor of prostaglandin synthetase, partially inhibits the endothelium-dependent relaxation and abolishes cyclic AMP accumulation; whereas, methylene blue partially inhibits the relaxation and abolishes cyclic GMP accumulation. All vessel responses are blocked by a combination of the two inhibitors (Ignarro, 1985). It is suggested that in the vascular endothelium arachidonic acid stimulates the synthesis and/or release of a relaxing factor (other than EDRF), as well as the synthesis and/or release of PGI₂. The relaxing factor and PGI₂ diffuse into the vascular smooth muscle to activate guanylate cyclase and adenylate cyclase respectively, leading to vascular relaxation.

*P. physalis* venom has been hypothesized to stimulate vasodilatory eicosinoid synthesis since sodium meclofenamate blocks its vasodilatory effect (Loredo *et al.*, 1986). The venom also stimulates endogenous phospholipase A₂ activity, resulting in increased arachidonic acid and prostaglandin levels (Shier, 1980). In addition, intravenous administration of piripost, a leukotriene inhibitor, reduces the cutaneous vascular leakage accompanied exposure to the venom (Burnett and Calton, 1987). This implies that an increased arachidonic acid level is associated with the increased leukotriene activity. Thus, the vasodilatory effect of *P. physalis* venom may result from activation of endogenous phospholipase A₂ activity to produce arachidonic acid which can elevate cyclic AMP and cyclic GMP by providing substrates for synthesis of PGI₂ and a relaxing factor (an unknown agent other than EDRF). Stimulation of vasodilatory
prostaglandin synthesis by the venom, however, appears to be fundamental or even exclusive since the vascular relaxation is not attenuated when guanylate cyclase is inhibited. In this situation arachidonic acid is shunted to the pathway for the synthesis of a vasodilatory ecosinoid.

Direct effects of P. physalis venom on vascular smooth muscles

It was hypothesized that P. physalis venom increased cell membrane permeability to certain monovalent and divalent cations by creating holes in the membrane (Burnett and Calton, 1987). Calcium ion is one candidate. The influx of calcium may in turn induce phospholipase A₂ and/or prostaglandin synthetase (including cyclooxygenase and endoperoxidase) activity. Although pre-treatment of arterial rings with quinacrine, a weak phospholipase A₂ inhibitor, did not reduce the effect of the venom according to Loredo et al. (1986), the possibility of activation of this enzyme by the venom has not been ruled out.

Azuma et al. (1986) reported that venom from a jellyfish, Carybdea rastonii, produced tonic contraction in rabbit aortic strips. That was drastically reduced in calcium-free medium and by verapamil and diltiazem. The stimulated calcium influx of the strip by the venom, associated with the contraction, was markedly attenuated by verapamil, suggesting an extracellular calcium-dependent actions of Carybdea venom.

In this study P. physalis venom induces vascular constriction in KCl-depolarized arterial rings regardless of the presence or absence of an intact endothelium. Similar to the action of Carybdea venom, the endothelium-independent, direct contractile effect of P. physalis venom may be linked to the increased membrane permeability to extracellular calcium ions as that hypothesized earlier. Depolarization of the smooth muscle by KCl to a
particular threshold level may facilitate the opening of voltage-dependent calcium channels, thus potentiating the action of *P. physalis* venom. When the effect of increased calcium influx in vascular smooth muscle predominates such as when the endothelium is absent, or in KCl depolarized rings, contraction occurs. However, when the effect of increased calcium influx in the endothelium predominates, such as in norepinephrine precontracted rings, then endogenous phospholipase A<sub>2</sub> is overwhelmingly stimulated and relaxation occurs.

Azuma *et al.* (1986) also reported that Carybdea venom significantly increased $^3$H efflux from $[^3$H]-norepinephrine preloaded aortic strips which was completely inhibited in calcium-free medium. The incorporation of $[^3$H]-norepinephrine to the presynaptic membrane was also reduced in the presence of Carybdea venom. These adrenergic potentiation actions were associated with the increased calcium influx in the aortic strips.

In addition to its action on smooth muscle cell membrane *P. physalis* venom may also stimulate calcium influx in adrenergic nerve endings thereby potentiating its action on the smooth muscle. In this study adrenergic inhibitors such as dibenzyline (a mixed $\alpha$ receptor blocking agent) and yohimbine (an $\alpha_2$ receptor blocking agent, which also blocks $\alpha_1$ receptor at higher doses) inhibit the contraction caused by the venom. Activation of $\alpha_1$ receptors increases calcium influx from the extracellular space and its release from the intracellular stores while activation of $\alpha_2$ receptors exclusively increases extracellular calcium influx (Nichols and Ruffolo, 1988; and Van Zwieten and Timmermans, 1988). Increased permeability to calcium in adrenergic presynaptic endings facilitate the release of norepinephrine, and in postsynaptic end plates the contractile response.
Therefor, adrenergic potentiation by *P. physalis* venom may play a role in vascular smooth muscle contraction.

PGF$_{2\alpha}$ raises intracellular calcium levels both by increasing calcium influx from the extracellular and by stimulating its release from intracellular stores (Inoue *et al.*, 1988), and thus facilitates the contractile action of norepinephrine (Aristegui and Enero, 1988). The vasoconstriction caused by PGF$_{2\alpha}$ is mediated by a proposed receptor mediated mechanism (Miwa *et al.*, 1988). *P. physalis* venom may increase the membrane permeability of smooth muscle to calcium, thereby potentiate the constriction in PGF$_{2\alpha}$ precontracted, intact rings in the present study.

*P. physalis* venom produces constriction in rings with low passive tension but not those under high tension induced by adrenergic agonists. High tension may have masked the direct action of the venom on the smooth muscle and intramural nerve ending whereas low tension maximized the effect.

**Rat Atrial Preparations**

In rat atrial preparations, *P. physalis* venom has a similar behavior to that of previous experiments on rabbit atrial preparations (Bonlie *et al.*, 1988). At a low (0.5 mM) extracellular calcium concentration no significant effect is observed. At normal or higher extracellular calcium concentrations (2.5 mM or higher) the positive inotropic effect produced by the venom is dose-dependent. When compared with those of rabbit atrial preparations, the slopes of the dose response curves at different extracellular calcium concentrations are less steep. This may result from the variation in reactivity of different animal species; nevertheless, this study dose confirm the
previous finding that the positive inotropic effect of *P. physalis* venom is dependent on extracellular calcium.

**Cultured Embryonic Chick Heart Cells**

*Calcium influx*

The inhibition of the Na+, K+-pump and generalized cell membrane depolarization has been suggested as possible mechanisms for the positive inotropic effect of *P. physalis* venom (Larsen and Lane 1966). The influence of the venom on cardiac transmembrane fluxes of crucial ions such as sodium, calcium and potassium under have not been examined, however.

Data from this study shows that *P. physalis* venom markedly increases calcium influx of cultured embryonic chick heart cells. The shape of the dose response curve implies a ligand-receptor relationship. The increased calcium influx caused by the venom is completely abolished by such dihydropyridine (DHP) calcium channel blockers, as diltiazem and verapamil. Venom-induced activation of calcium channels is indicated as the underlying mechanism of the positive inotropic effect of the venom. This finding supports the reported clinical effectiveness of verapamil in treating intoxication caused by *P. physalis* venom (Burnett *et al.*, 1985).

In Bonlie's (1988) study, the positive inotropic effect of *P. physalis* venom on rabbit atrial preparations was partially blocked by diltiazem. The incomplete inhibition observed by him might result from the inability of diltiazem to penetrate sufficiently into a large chunk of tissue.

In the heart and vascular smooth muscles there are two types of voltage-dependent calcium channels designated as T and L (Benham *et al.*, 1987; Godfraind *et al.*, 1988; Hess, 1987; Kawano and DeHaan, 1989; Tsien *et
al., 1987; and Wang et al., 1989). In embryonic chick heart cells the L type channel is fully activated at -30 mV and exhibits midpoint inactivation at -10 mV with a mean opening time of less than 1 ms for mode 1 gating or 20 ms for mode 2 gating. The L type channel is classically sensitive to DHP calcium channel blockers. In contrast, the T type channel is fully activated at -80 mV and totally inactivated at -30 mV with mid-point inactivation at -53 mV and a mean opening time of 1-2 ms. Furthermore the T type channel is insensitive to DHP calcium channel blockers; however, Ni^{2+} reduces its current by 13% (Hess, 1987). A much greater density of the T type channels than the L type has been found in the developing embryonic chick heart, so that the T type channels conduct the majority of the current (Kawano and DeHaan, 1989).

In this study, the increased calcium influx produced by *P. physalis* venom is completely inhibited by diltiazem and verapamil. This may indicate that the venom elevates calcium influx by increasing the opening of L type channels. The calcium influx of the control group, however, is not affected by diltiazem and verapamil because the T type current is dominant in embryonic chick heart cells and insensitive to DHP calcium channel blockers.

*Sodium influx*

Sodium influx of cultured embryonic chick heart cells is only moderately increased in the presence of *P. physalis* venom when compared with calcium influx. Neither flecainide (a sodium channel blocker) nor verapamil has a significant inhibitory effect on the increased sodium influx caused by the venom.

Recent research on ionic channels favors a multi-ion pore model for
calcium channels (Tsien et al., 1987). It was shown that a large sodium influx passed through cardiac calcium channels in the virtual absence of external calcium. The calcium channel-mediated sodium influx was blocked more than 50% by the addition of 1.3 μM extracellular calcium. A graded sodium current reduction of calcium channels with increasing extracellular calcium concentration was consistent with calcium binding to a site with a dissociation constant of about 1 μM. The calcium binding site had a high affinity for calcium and a low affinity for sodium (Tsien et al., 1987).

At normal physiological calcium concentrations, however, sodium current through calcium channels is negligible. It is possible that under the influence of P. physalis venom, an increased calcium influx is accompanied by a noticeably increased sodium influx in the same calcium channel. In this study verapamil, which is able to completely block the increased calcium influx caused by the venom, does not inhibit the increased sodium influx to a significant degree. For this reason, it is not likely that P. physalis venom increases sodium influx through calcium channels.

There is evidence of homology between sodium and calcium channels (Henry, 1983; Rosenberger and Triggle, 1978). It has been known for a long time that DHP calcium channel receptor blockers from the phenylalkylamine class such as verapamil, and from the benzothiazepine class such as diltiazem, also act on sodium channels (Henry, 1983; Rosenberger and Triggle, 1978). DHP calcium channel blockers have been reported to antagonize both calcium and sodium channels in a voltage-dependent manner (Yatani and Brown, 1985; Yatani et al., 1986a and 1986b). BAY-K
8644 (a DHP calcium channel blocker) has been shown to exist as a stereoisomeric pair. The (-) form had a pronounced agonist effect on L type calcium channels while the (+) form an antagonist effect. Similar effects of the two forms of BAY-K 8644 were seen in single sodium current (Yatani et al., 1988). The primary structure of the DHP calcium channel receptor in skeletal muscle was reported to be homologous to peptide sequence in sodium channels from a variety of species (Tanabe et al., 1987). It has been suggested that perhaps the DHP receptor domain is encoded by a gene that has reduplicated and is part of both sodium and calcium channels (Yatani et al., 1988).

Increased calcium and sodium influxes under the influence of *P. physalis* venom may implicate its effect on DHP receptors of both channels. In this study flecainide, a sodium channel blocker, however, has no effect on the sodium influx of either the control group or the venom treated group. The blocking action of this agent may be too weak because the concentration used is not high enough. Nevertheless, the speculation that *P. physalis* venom may increase sodium influx by fast sodium channels still can not be proved. Whether *P. physalis* venom modulates sodium channels or alters sodium current of calcium channels needs to be further explored by single cell or single channel electrophysiological studies. Using a more powerful sodium channel blocker such as tetrodotoxin (TTX) to inhibit the increased sodium influx caused by the venom may also be helpful.

It was reported that in the embryonic chick heart sodium/calcium exchange was much more developed than the active calcium transport system (Vetter and Will, 1986; and Vetter et al., 1986). Alterations of intracellular and extracellular calcium, sodium, pH and membrane potential resulted
in change in calcium and sodium contents that were consistent with sodium/calcium exchange. If the exchange mechanism was operative and not kinetically limited, responses of the cell to restore homeostasis were predictable. The thermodynamic equilibrium for sodium/calcium exchange could be expressed as 
\[(Na_i/Na_o)^n \exp[(n-2)E_mF/RT] = Ca_i/Ca_o\]
(where: \(Na_i\) = intracellular Na; \(Na_o\) = extracellular Na; \(n\) = the number of Na ions exchange for 1 Ca; \(E_m\) = membrane potential; \(R\) = gas constant; \(T\) = absolute temperature; \(F\) = Farady constant; \(Ca_i\) = intracellular free calcium; and \(Ca_o\) = extracellular calcium). Sodium efflux measurement in cultured embryonic chick heart cells revealed a large ouabain-insensitive component, one-third of which was inhibited by removal of extracellular calcium. Incubating the cell in sodium-free solution led to 1.5 to 2 fold increase in intracellular calcium that remained elevated for at least 15 minutes. The excessive cytoplasmic calcium was taken up by the sarcoplasmic reticulum and mitochondria. Cells became maximally loaded with calcium after exposure to extracellular sodium level of less than or equal to 20 mM. The loading fell off sharply when the sodium level was raised to more than 20 mM. The movement of sodium against its electrochemical gradient was associated with calcium accumulation. During Na+-K+ pump inhibition by 10^-4 M ouabain, lowering the extracellular calcium concentration from 1.25 to 0.75 mM caused a 26% elevation of intracellular sodium. In contrast, raising extracellular calcium from 1.25 to 2.7 mM resulted in a 25% fall of intracellular sodium against its electrochemical gradient (Jacob et al. 1987; and Murphy et al., 1986).

In this study, *P. physalis* venom increases calcium influx 627% per minute and sodium influx 122% per minute maximally in cultured
embryonic chick heart cells. The maximal increases occur both at venom concentration of 2 µg/ml. Since sodium/calcium exchange is the major mechanism to restore cellular homeostasis by the embryonic chick heart, a large calcium influx accelerates the operation of this mechanism to eliminate excessive cytoplasmic calcium. As predicted by the equation, 
\[(Na_i/Na_o)^{n_{exp}[(n-2)E_mF/RT]} = Ca_f/Ca_o\], elevating \(Ca_f\) will cause a rise in \(Na_i\). For this reason, an increased calcium influx is inevitably associated with an increased sodium influx in embryonic chick heart cells, reflecting the indirect effect of the venom (Fig 27). There are also other mechanisms to restore cytoplasmic free calcium level, such as uptake by mitochondria, sequestration by sarcoplasmic reticulum and plasmic membrane. These processes, however, are far less developed in the embryonic chick heart (Vetter and Will, 1986; and Vetter et al., 1986), as well as more expensive energy wise. In addition to its indirect effect on accelerating sodium/calcium exchange by increasing calcium influx, \(P.\ physalis\) venom may have a direct activating effect on sodium/calcium exchange. It is observed in this study that the increased sodium influx is not significantly affected when the increased calcium influx caused by the venom is completely blocked by 0.01 M verapamil.

**Rubidium influx**

Rubidium is an analogue of potassium (Segal et al., 1977; Frelin et al., 1986; Otero and Szabo, 1988; Vormanen, 1984). Rubidium influx of cultured embryonic chick heart cells is not influenced by \(P.\ physalis\) venom at lower dose levels. A very high concentration of the venom, however, reduces the influx of rubidium.
Fig. 27 Summary diagram of ionic fluxes under the influence of *P. physalis* venom. The bilayer circle represents an embryonic chick heart cell. Bold arrows indicates increased fluxes. a. calcium channel. b. calcium-dependent potassium channel. c. sodium/calcium antiport. d. Na⁺/K⁺ pump.
Potassium influx is an active transport process mediated by the voltage-insensitive Na+/K+-pump against an electrochemical gradient (Bahinski et al., 1988). Intra-and extracellular sodium and potassium concentrations modify the activity of the pump (Nakao and Gadsby, 1989). It was shown that 5.4 mM extracellular potassium and 50 mM intracellular sodium (and 10 mM ATP) strongly activated the pump in a sigmoidal curve manner with a steep positive slope between about 0 and -100 mV in guinea pig heart cells (Gadsby and Nakao, 1989). It has been further reported that intracellular sodium exerts a long-term modulating effect on the number of physiologically functional Na+/K+-pump sites (Kim and Smith, 1986c).

Ianuzzo and Dabrowski (1987) suggested that different aerobic capacities in striated muscle were reflective of different contractile activity which imposed greater demands on sarcolemmal ion translocation and might thus set ion pumping capacities. They found that the highest and the lowest Na+, K+-pump activity was in the heart and white gastrocnemius muscle respectively. A linear correlation between Na+/K+-pump activities and succinate dehydrogenase activity was found in the six different types of muscle studied in rat. It was concluded that Na+/K+-pump activity had a high positive correlation with aerobic capacity in striated muscles.

In this study P. physalis venom significantly increases sodium influx which would supposedly stimulate the Na+/K+-pump. However, only until very high venom levels are reached is rubidium influx decreased, indicating inhibition of the Na+/K+-pump. Perhaps an enormous calcium influx accompanied by increased contraction and consequential utilization of ATP leads to energy depletion. Energy crises may result in inhibition of the Na+/K+-pump. Measurement of intracellular ATP at different doses of
the venom could further test this hypothesis.

Rubidium efflux

In this study rubidium efflux of cultured embryonic chick heart cells is significantly increased under the influence of *P. physalis* venom. The increased rubidium efflux is dose-dependent and appears to be highly exponentially correlated with the increased calcium influx produced by the venom.

Potassium efflux is a process of passive diffusion through multiple types of potassium channels which lack specific ligands interacting with them (Cook, 1988). In the heart three types of potassium channels, sodium-activated, ATP-sensitive and calcium-dependent potassium channels have been identified. It has been reported that DHP calcium channel blockers do not affect potassium current as they do for sodium current. Functionally equivalent DHP receptors present in calcium and sodium channels are not present in potassium channels (Yatani et al., 1988).

The sodium-activated potassium channel has been described as a novel class of ionic channel in the mammalian heart. It is voltage-dependent and activated when intracellular sodium concentration is more than 20 mM (Kameyama et al., 1984). ATP-sensitive potassium channels are reported to be activated by depletion of ATP and inhibited by ATP or substrates for ATP production through glycolysis, mitochondrial oxidative phosphorylation or the creatine kinase system (Weiss and Lamp, 1989; Noma and Shibasaki, 1985). Exposure of cultured chick heart cells to cyanide and 2-deoxy-D-glucose causes marked increases in cell sodium (sevenfold) and calcium (fivefold), and a decreased potassium content (one fifth normal). Metabolic inhibition of ATP production could result in
persistent abnormalities in potassium homeostasis associated with functional abnormalities (Ishida et al., 1988).

Calcium-dependent potassium channels were reported to be stimulated by high intracellular calcium concentrations (Kitamura et al., 1989; Klockner et al., 1989; Blatz and Magleby, 1984). Fluoride intoxication caused increased intracellular calcium and triggered calcium-dependent potassium efflux, leading to hyperkalemia and arrhythmia (Cumming and McIvor, 1988). Extracellular calcium induced an immediate rise in potassium efflux of human red blood cells pretreated with low-ionic-strength solution. The increased potassium efflux was inhibited by oligomycin and high external potassium. It was suggested that a low-ionic-strength solution exposed calcium-sensitive sites of the potassium channel to the external medium (Adorante and Macey, 1986).

*P. physalis* venom causes a marked increase in calcium influx of cultured embryonic chick heart cells. Increased intracellular calcium levels may stimulate internal calcium-sensitive sites of calcium-dependent potassium channels, resulting in increased potassium (rubidium) efflux (Fig. 27). The high correlation between the influx of calcium and the efflux of rubidium may indicate the operation of such a mechanism. In the presence of high levels of *P. physalis* venom, a high calcium influx associated with strong contraction may lead to ATP depletion. This is substantiated by the inhibition of the Na+/K+-pump at high venom levels. Under such a condition activation of ATP-sensitive potassium channels may also contribute to the increased rubidium efflux.

2-deoxy-D-glucose uptake and trypan blue staining

In the literature it was suggested that *P. physalis* venom created
holes in the cell membrane, allowing monovalent and divalent cations to pass through (Burnett and Calton, 1987). However, the size and the specificity of the hypothetical hole were unknown. In this study trypan blue, a large molecule, was unable to cross the cell membrane under the influence of the venom, so the hole should be at least smaller than a trypan blue molecule.

Glucose is a smaller molecule than trypan blue. 2-Deoxy-D-glucose is an analog of glucose and enters the cell by the same mechanism. Since it is not metabolized by the cell, it is a good indicator for measurement of glucose uptake (Segal et al., 1977 and Gordon et al., 1986). This study shows that uptake of 2-deoxy-D-glucose by cultured embryonic chick heart cells is not altered by P. physalis venom. Only at an extremely high concentration of the venom (20 μg/ml) is the uptake of 2-deoxy-D-glucose significantly reduced. This indicates that the hypothetical hole large enough to allow a glucose molecule to pass though does not exist.

The decreased 2-deoxy-D-glucose uptake may be a sign of a disturbance of glucose metabolism. Calcium ion is an important enzymatic regulator of some important steps in the glucose metabolic pathway. First, the activation of phosphorylase b (an inactive form) to phosphorylase a (an active form) is regulated by Ca²⁺-dependent protein kinase. The active phosphorylase breaks down glycogen to glucose and increases glucose utilization from intracellular stores. Secondly, Ca²⁺-dependent protein kinase phosphorylates 6-phosphofructose 2 kinase (PFK2) to an inactive form, resulting in decreased production of fructose-2,6-diphosphate which is the key stimulator of phosphofructose kinase, the rate limiting enzyme of glycolysis. In addition, pyruvate kinase, an enzyme that transforms
pyruvate to acetyl CoA, uses potassium ion as a co-factor.

High concentrations of *P. physalis* venom markedly increases calcium influx and potassium efflux. It is speculated that excessive cytoplasmic calcium may impede glucose metabolism, mainly in the key steps of glucose metabolic pathway mentioned above. Activation of Ca$^{2+}$-dependent protein kinase increases breakdown of glycogen. More importantly, activation of the kinase decreases production of phosphofructose 2, 6 di-phosphate, leading to a shut down of glycolysis by blocking the enzymatic transformation of 6-phosphofructose to phosphofructose-1,6-diphosphate. Decreased intracellular potassium levels due to an increased potassium efflux and inhibition of Na$^+$/K$^+$ pump (only at high venom concentrations) caused by the venom make less co-factor available to pyruvate kinase, further limiting glucose utilization. Severe inhibition of glucose catabolism and increased breakdown of glycogen result in intracellular glucose accumulation. For this reason, glucose uptake of the cell decreases. Nevertheless, the measurement of glycolytic and glycogenolytic enzyme activities at high venom concentrations need to be performed to prove above speculations.

Conclusions

1. *P. physalis* venom produces significant vascular relaxation in endothelium-intact rabbit femoral arterial rings precontracted by adrenergic agonists. This action does not depend on EDRF. The data support the hypothesis that the venom stimulates vasodilatory prostaglandin synthesis in vascular endothelium probably by activating phospholipase A$_2$ and/or prostaglandin synthetase.
2. *P. physalis* venom significantly produces vascular constriction in KCl depolarized rings regardless of the presence or absence of an intact endothelium. The constriction is completely inhibited by dibenzyline, a mixed α receptor blocker, and markedly attenuated by yohimbine, an α2 receptor blocker. The venom may increase membrane permeability to calcium, leading to adrenergic potentiation and smooth muscle contraction.

3. *P. physalis* venom has a dose-dependent positive inotropic effect on rat atrial preparations, which is directly related to extracellular calcium concentrations. At high venom levels, arrhythmias occur.

4. *P. physalis* venom markedly increases calcium influx through L type calcium channels of cultured embryonic chick heart cells. This action is dose-dependent and is completely inhibited by diltiazem or verapamil.

5. Sodium influx of cultured embryonic chick heart cells is significantly increased under the influence of *P. physalis* venom. The increased sodium influx is much less than the increased calcium influx. Flecainide, a sodium channel blocker, and verapamil, a calcium channel blocker do not inhibit the increased sodium influx of the venom. The elevated sodium influx may result from the increased sodium/calcium exchange which is the major mechanism for the embryonic chick heart to eliminate excessive cytoplasmic free calcium.

6. *P. physalis* venom has no effect on rubidium influx of cultured embryonic chick heart cells at lower dose levels. Only at a high venom concentration (6 μg/ml) is the influx of rubidium decreased,
indicating inhibition of the Na+/K+-pump. This appears to be an indirect effect of the venom. Depletion of ATP associated with greatly increased contractility of the heart cells accompanied by an elevated calcium influx may contribute to the inhibition of the pump.

7. Rubidium efflux of cultured embryonic chick heart cells is significantly increased in the presence of *P. physalis* venom. The increased rubidium efflux is highly exponentially correlated with the increased calcium influx. Stimulation of calcium-dependent potassium channels by a large calcium influx may play an important role. Activation of ATP-sensitive potassium channels in ATP depletion at high venom concentrations may also contribute to the increased rubidium efflux.

8. The uptake of 2-deoxy-D-glucose by cultured embryonic chick heart cells is not modified by *P. physalis* venom. Only at an extremely high concentration (20 μg/ml) of the venom is the uptake of the glucose decreased. Excessive cytoplasmic free calcium resulting from a markedly increased calcium influx may interfere with glucose metabolism and transport by influencing the activities of important enzymes.

9. There is no evidence that *P. physalis* venom creates non-specific holes large enough to allow trypan blue or a 2-deoxy-D-glucose molecule to cross the membrane of cultured embryonic chick heart cells.

10. *P. physalis* venom appears to have a positive completeness of contraction effect on beating cultured embryonic chick heart cells. The magnitude of the action is correlated with the increased calcium influx.
At high venom concentrations (> 2 μg/ml) arrhythmias, flutter, fibrillation and finally asystole occur.
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