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CARDIOVASCULAR ACTIONS OF THE VENOM FROM THE IRUKANDJI (*CARUKIA BARNESI*) JELLYFISH: EFFECTS IN HUMAN, RAT AND GUINEA-PIG TISSUES *IN VITRO AND* IN PIGS *IN VIVO*

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SUMMARY

1. We have investigated the cardiovascular pharmacology of the crude venom extract (CVE) from the potentially lethal, very small carybdeid jellyfish *Carukia barnesi*, in rat, guinea-pig and human isolated tissues and anaesthetized piglets.

2. In rat and guinea-pig isolated right atria, CVE (0.1–10 μg/mL) caused tachycardia in the presence of atropine (1 μmol/L), a response almost completely abolished by pretreatment with tetrodotoxin (TTX; 0.1 μmol/L). In paced left atria from guinea-pig or rat, CVE (0.1–3 μg/mL) caused a positive inotropic response in the presence of atropine (1 μmol/L).

3. In rat mesenteric small arteries, CVE (0.1–30 μg/mL) caused concentration-dependent contractions that were unaffected by 0.1 μmol/L TTX, 0.3 μmol/L prazosin or 0.1 μmol/L ω-conotoxin GVIA.

4. Neither the rat right atria tachycardic response nor the contraction of rat mesenteric arteries to CVE were affected by the presence of box jellyfish (*Chironex fleckeri*) antivenom (92.6 units/mL).

5. In human isolated driven right atrial trabeculae muscle strips, CVE (10 μg/mL) tended to cause an initial fall, followed by a more sustained increase, in contractile force. In the presence of atropine (1 μmol/L), CVE only caused a positive inotropic response. In separate experiments in the presence of propranolol (0.2 μmol/L), the negative inotropic effect of CVE was enhanced, whereas the positive inotropic response was markedly decreased.

6. In anaesthetized piglets, CVE (67 μg/kg, i.v.) caused sustained tachycardia and systemic and pulmonary hypertension. Venous blood samples demonstrated a marked elevation in circulating levels of noradrenaline and adrenaline.

7. We conclude that *C. barnesi* venom may contain a neural sodium channel activator (blocked by TTX) that, in isolated atrial tissue (and *in vivo*), causes the release of transmitter (and circulating) catecholamines. The venom may also contain a 'direct' vasoconstrictor component. These observations explain, at least in part, the clinical features of the potentially deadly Irukandji syndrome.

Key words: *Carukia barnesi*, catecholamines, Irukandji syndrome, jellyfish, neuronal sodium channel, noradrenaline, sympathetic nervous system, venom.

INTRODUCTION

The Irukandji syndrome is a potentially lethal complex of clinical signs and symptoms that has been attributed to various carybdeid jellyfish stings (for a review, see Burnett *et al.*[1]). The syndrome is named after an Australian aboriginal tribe that inhabited the Palm Cove region of north Queensland where this envenomation was commonly reported.[2,3] However, the syndrome has also been described throughout the Pacific basin[1] and offshore Florida,[4] with Irukandji-like stings also reported from southern Australia[5] and Hawaii.[6] The syndrome has three recognized clinical forms or patterns consisting of: (i) acute pain; (ii) catecholamine-like effects; and (iii) delayed complications relating to cardiopulmonary decompensation.[1–3,7–10]

The initial sting is itself typically mild and is followed by a characteristic delay before the onset of systemic effects 5–40 min later.[1–3,10] These begin with severe low back pain, muscle cramps in the legs, abdomen, chest and upper limbs, and nausea and vomiting. Other classical early features include excessive sweating, which may be localized or generalized, agitation, anxiety, tachycardia and often headache. Hypertension and supraventricular tachyarrhythmia also commonly occur. Occasionally, the hypertension may be life threatening in severity, as seen in two recent Irukandji syndrome fatalities associated with intracerebral haemorrhage.[11] Less commonly, the illness is complicated by pulmonary oedema, toxic heart failure[7,8] or cerebral oedema.[12] The signs and symptoms may last from hours to several days and usually require hospitalization.[9]

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Although first described 50 years ago,[2,13] the pharmacological basis of the syndrome has been elusive. To date, only a single species of jellyfish, namely *Carukia barnesi*, has definitely been shown to be capable of causing the syndrome,[3,10] although others are suspected.[1,7,14,15] Unlike the larger and better known major 'box' jellyfish *Chironex fleckeri*, which has a bell of up to 30 cm and up to 60 tentacles (each up to 3 m in length), *C. barnesi* is almost invisible to the naked eye with its pyramidal-shaped bell being only 1–2 cm in diameter and its four tentacles extending only to 35 cm in length.[1,16,17] Consequently, few *C. barnesi* specimens have been captured, thereby limiting investigation of its venom.

As part of our investigation of the Irukandji syndrome, we recently developed a rapid and simple method to extract active cubozoan jellyfish venom.[18] After two highly successful collecting seasons (the summers of 1999–2000 and 2001–2002), we obtained sufficient jellyfish to allow the first *in vitro and in vivo* analyses of the cardiovascular activity of *C. barnesi* venom extract. Some of this work has been presented previously in abstract form.[19–22]

METHODS

Jellyfish capture and identification

All *C. barnesi* jellyfish were captured immediately off coastal beaches between Cairns and Port Douglas, north Queensland, by daily netting during late December–early January 1999–2000 and 2001–2002. The net was specifically designed with a 2 mm cut-off for this purpose. Specimens were placed immediately into vessels containing seawater pending light microscopic examination. Those specimens identified as *C. barnesi* by gross morphology[16] were snap-frozen in plastic bags and transported, by air, to Melbourne at –70°C and stored at –70°C prior to processing. Specimens were examined by light microscopy and formally identified, prior to processing, as *C. barnesi* according to Southcott's description[16] of bell, tentacle and nematocyst morphology. Specimens of *C. fleckeri* were collected offshore from Darwin in the Northern Territory during January–February 1999–2000 and formally identified by Dr P Alderslade of the Northern Territory Art Gallery and Museum. The tentacles were removed from the jellyfish and frozen at –70°C prior to transport by air, still frozen, to Melbourne.

Venom extract preparation and protein determination

Crude venom extract (CVE) was obtained from pooled adult specimens of *C. barnesi* in consecutive batches using a rapid mortar and pestle approach similar to that described previously.[18] This method was modified for extraction of venom from *C. fleckeri* nematocysts. The protein content of each CVE was determined by a colourimetric protein assay kit (Pierce, Rockford, IL, USA).

Irukandji jellyfish

Briefly, in a typical experiment 20–30 pooled whole, thawed, adult *C. barnesi* specimens were placed in a prechilled siliconized glass mortar and pestle containing 3 mL chilled phosphate-buffered saline (PBS) and ground for 15 min surrounded by an ice slurry. The resulting mixture was then centrifuged at 2292 *g* for 15 min at 5°C. The supernatant was removed, protein content estimated and samples aliquoted and stored at –70°C until required.

Box jellyfish

To prepare mortar-and-pestle-ground *C. fleckeri* nematocyst CVE, 20 g box jellyfish tentacle was washed with 35 mL cold PBS for 10 min. The contents were then poured onto a plastic tray and the tentacles scraped with forceps to recover the nematocysts. The tentacles were removed and the remaining mixture was centrifuged at 52 *g* for 10 min at 5°C. The resulting pellet was resuspended in 3 mL PBS, transferred to a prechilled siliconized glass mortar and pestle and ground for 15 min. The resulting mixture was then centrifuged at 2292 *g* for 15 min at 5°C. The supernatant was removed, protein content estimated and samples aliquoted and stored at –70°C until required.

In vitro analysis

The pharmacological activity of CVE was analysed *in vitro* using guinea-pig and rat isolated left and right atria, rat isolated mesenteric artery and human right atrial trabeculae preparations. Experiments on animal tissues were undertaken with the approval of the University of Melbourne Animal Experimentation and Ethics Committee, whereas human heart experiments were approved by the Prince Charles Hospital Human Ethics Committee. Male Sprague-Dawley rats (250–450 g) or male Hartley guinea-pigs (300–500 g) were anaesthetized in a mixture of CO_2 (80%) and O_2 (20%) for up to 2 min before exsanguination. Tissues were excised rapidly and bathed in Krebs' physiological salt solution (PSS) with the following composition (in mmol/L): NaCl 119; KCl 4.69; $MgSO_4{\cdot}7H_2O$ 1.17; KH_2PO_4 1.18; glucose 11; $NaHCO_3$ 25; $CaCl_2{\cdot}6H_2O$ 2.5; EDTA 0.026. The PSS was saturated with carbogen O_2 (95%) and CO_2 (5%) at pH 7.4 (unless stated otherwise).

Guinea-pig and rat left and right atria

Spontaneously beating right atria were dissected free and pierced by two 30 G hooks, one of which was hooked through a static platinum loop embedded in an acrylic organ bath leg; the other was hooked through a stainless-steel wire loop terminating at a Grass FTO3C isometric force transducer (Grass Instruments, Warwick, RI, USA). Electrode legs were fixed to a laterally constrained stage, which was raised or lowered vertically by a micrometer (Mitutoyo Manufacturing, Tokyo, Japan). Tissues were suspended in a 25 mL jacketed organ bath containing PSS saturated with carbogen at 37°C and were then stretched to a passive force that caused an optimal active inotropic response. Left atria were stimulated electrically via two punctate platinum electrodes (1 Hz, 0.5 msec) at 150% of threshold voltage. Both spontaneous and electrically driven inotropic responses were measured via a six-channel transducer amplifier (no. 108; Baker Medical Research Institute, Melbourne, Victoria, Australia) with the spontaneous right atrial period measured by a period meter (no. 173; Baker Medical Research Institute) triggered by the inotropic response. All responses were recorded on a six-channel Neotrace flat bed chart recorder (Neomedix, Sydney, NSW, Australia). Responses were calculated as change in atrial rate (b.p.m.) and increase over basal contraction force (%) in right and left atria, respectively.

Atria were equilibrated for 30 min after two exchanges of PSS and then incubated for 10 min with either atropine (1 μmol/L), propranolol (1 μmol/L) or box jellyfish antivenom (92.6 units/mL). In the case of tetrodotoxin (TTX; 0.1 μmol/L), incubation was for 30 min. The *C. barnesi* or *C. fleckeri* CVE was then added to each bath in increasing cumulative concentrations (expressed as μg crude extract protein/mL bath volume), allowing sufficient time between additions for any change in response to reach plateau. In experiments with propranolol and *C. barnesi* CVE, with the highest concentration of venom present, TTX (0.1 μmol/L) was added to each bath for a minimum of 10 min.

Rat small mesenteric artery

Segments of the third-order branch of the mesenteric artery (2 mm length) were dissected out and mounted on 40 μm diameter stainless-steel wires in a double-chamber Mulvany-Halpern-style isometric myograph (JP Trading, Aarhus, Denmark). Chambers were filled with PSS (described above) with half-normal PSS glucose (5.5 mmol/L) and saturated with carbogen (95% O_2/5% CO_2) at pH 7.4. Tissues were adjusted to a passive tension equivalent

to a transmural pressure of 90 mmHg as generated by a passive length–tension curve and equilibrated for 30 min.[23]

A contraction to potassium depolarizing solution (KPSS; 2 min exposure to PSS with an equimolar substitution of KCl for NaCl; K^+ 124 mmol/L) was performed to assess vessel viability and to provide a reference contraction. This was followed by exposure to 10 μmol/ noradrenaline L with subsequent washout and equilibration. Vessels were then incubated with one of TTX (0.1 μmol/L), ω-conotoxin GVIA (0.1 μmol/L), prazosin (0.3 μmol/L), box jellyfish antivenom (92.6 units/ mL) or vehicle (H_2O) for 10 min before the vessel was actively precontracted with sufficient arginine vasopressin (AVP; approximately 0.1 nmol/L) to elicit a small contraction equivalent to between 5 and 10% of the maximum contraction to KPSS. Pre-activation with AVP was necessary in order to unmask the contractile effects of weak vasoconstrictor agents.[24] *Carukia barnesi* CVE was then added to each bath in increasing cumulative concentrations (expressed as μg crude extract protein/mL bath volume), allowing sufficient time between additions for any change in response to reach plateau. Contractions were expressed as %KPSS maximum contraction.

Human right atrium

Human right atrial appendages were obtained from five patients (average age 50 ± 3 years) undergoing coronary artery bypass surgery at The Prince Charles Hospital. Drugs administered chronically prior to surgery included β-adrenoceptor antagonists ($n = 3$; metoprolol), L-type calcium channel blockers ($n = 1$; diltiazem), angiotensin-converting enzyme inhibitors ($n = 3$), hypolipidaemic agents ($n = 4$; atorvastatin or simvastatin), hypoglycaemic agents ($n = 1$), vasodilators (nitrates $n = 2$; nicorandil $n = 1$; prazosin $n = 1$) and diuretics ($n = 1$; indapamide). None of the patients was in heart failure. Upon surgical removal, right atrial appendages were placed immediately in sealed containers filled with ice-cold modified Krebs' PSS (mPSS; composition (in mmol/L): NaCl 98.5; KCl 5; $MgSO_4 \cdot 7H_2O$ 0.5; $NaHPO_4$ 1.0; $NaHCO_3$ 32; $CaCl_2 \cdot 2H_2O$ 2.25; EDTA 0.04; preoxygenated with carbogen (95% O_2/5% CO_2) at pH 7.4) and transferred to the laboratory. Trabeculae were dissected, mounted onto tissue-electrode blocks in 50 mL baths and electrically field stimulated (1 Hz, 5 msec duration, just over threshold voltage) as described previously.[25] A length–tension curve was constructed to determine the length at which maximal contractions occurred (L_{max}) and the tension adjusted to 50% L_{max} to decrease time-dependent decreases in basal force. The incubation medium was exchanged with mPSS containing, in addition, 5 mmol/L sodium fumarate, 5 mmol/L sodium pyruvate, 5 mmol/L sodium glutamate and 10 mmol/L glucose with or without atropine (1 μmol/L) or propranolol (0.2 μmol/L). The addition of 0.2 μmol/L propranolol would also block the effects of endogenously released noradrenaline, as we have observed previously with low concentrations of the β-adrenoceptor antagonist (–)-CGP 12177 and (–)-pindolol.[26,27] Following an incubation period of 60 min, tissues were exposed to 10 μg/mL *C. barnesi* CVE. Tissues were stable prior to the administration of CVE. For the 20 min immediately prior to CVE administration, changes in basal contractile force for the different groups were as follows: no antagonist, +1 ± 1%; 1 μmol/L atropine, –7 ± 2%; and 0.2 μmol/l propranolol, –3 ± 1% ($n = 5$ hearts). Therefore, correction factors for time-dependent changes were not applied to the effects of CVE on human atrial trabeculae. Recordings of contractile force were made on a 12-channel Watanabe recorder (Watanabe, Fujisawa, Japan).

In vivo analysis

The *in vivo* activity of CVE was examined in anaesthetized, mechanically ventilated piglets. The Royal Children's Hospital Animal Ethics Committee approved this study. General anaesthesia in piglets (bodyweight 4.5–6.8 kg) was induced by inhalation of halothane in O_2 and thereafter maintained by an initial bolus of propofol (10 mg/kg) followed by a constant of infusion at 10 mg/kg per h and morphine at 0.6 mg/kg per h. Mechanical ventilation via an endotracheal tube with 40% O_2 by an infant ventilator (BP 2001; Bear Medical Systems, San Diego, CA, USA) was set to maintain P_aCO_2 at 35–45 mmHg. Pulse oximetry (Biox 3700e; Ohmeda, Louiseville, CO, USA) with a probe attached to the tongue and the electrocardiograph (Electronics-for-Medicine, Honeywell, Pleasantville, NY, USA) were displayed continuously. Arterial blood gases and electrolyte measurements were performed at regular intervals. Maintenance fluid, consisting of 0.9% saline, was infused at 4 mL/kg per h into a peripheral vein.

Catheters were inserted into both femoral arteries, one to monitor arterial pressure and another to allow blood sampling. A catheter was inserted into the superior vena cava via a femoral vein to monitor central venous pressure. A Swan-Ganz thermodilution catheter (Model 93-132-5F; American Edwards Laboratories, Irvine, CA, USA) was inserted via the other femoral vein into a branch of the pulmonary artery with the aid of fluoroscopy. Systemic arterial blood pressure, pulmonary arterial pressure and heart rate were displayed on an Electronics-for-Medicine monitor and recorded on a paper chart recorder (Model 8K23H; Sanei Instruments, Tokyo, Japan). Cardiac output was measured by thermodilution (COM-2™ computer; Baxter Healthcare, Irvine, CA, USA). Injections of 0.9% saline at 0–5°C were made with a closed delivery system (CO-Set II; Baxter Healthcare). Pulmonary artery occlusion pressure and central venous pressure were measured at intervals.

Baseline blood samples for the measurement of plasma catecholamines were taken 15 min prior to i.v. injection of equal volumes (180 μL/kg) of vehicle (PBS) and, thereafter, *C. barnesi* CVE (67 μg/kg). Additional blood samples were collected at 10, 60 and 120 min after CVE administration. Blood samples were collected into ice-chilled tubes containing EGTA and glutathione. Plasma was separated by centrifugation and stored at –80°C until assayed for dihydroxyphenylalanine (DOPA), dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), noradrenaline, dihydroxyphenylglycol (DHPG) and adrenaline. Several samples were also analysed for creatine phosphokinase (including myocardial fraction).

Catecholamine assays

Serum catecholamine levels were determined from plasma with alumina adsorption, separated by HPLC and the amounts quantified by electrochemical detection, as described previously.[28] The chromatographic system consisted of a Model 480 high-precision pump, Gina autosampler, STH 585 column oven, Chromeleon 3.03 Chromatography Data System (all from Gynkotek, Germering, Germany), a 5100A colourimetric detector equipped with a 5021 conditioning cell and a 5011 analytical cell (all from Environmental Sciences Associates, Boston, MA, USA) and a 25 cm Altex Ultrasphere column (ODS 4.6 mm × 25 cm, 5 μm particle size; Beckman Instruments, Richmond, CA, USA).

Analysis was performed at 24°C with the operating potentials set at +0.35 V for the guard cell and –0.35 and +0.29 V for detectors 1 and 2, respectively. All measurements were made using the oxidizing potential applied at detector 2 and compounds in piglet serum were identified by their retention behaviour compared with that of authentic standard solutions. A sample of *C. barnesi* CVE was also assayed using this method to determine catecholamine levels.

Drugs

Drugs (and suppliers) were as follows: atropine SO_4 (Sigma, St Louis, MO, USA); Box Jellyfish Antivenom (CSL, Parkville, Victoria Australia); ω-conotoxin GVIA (Auspep, Parkville, Victoria Australia); noradrenaline ((–)-Arterenol bitartrate; Sigma); prazosin HCl (Sigma); propranolol HCl (Sigma); and TTX (Sapphire Bioscience, Sydney, NSW, Australia). Aliquots of CVE (crude venom extract), TTX (0.1 mmol/L) and ω-conotoxin GVIA (0.1 mmol/L) were stored until use at –70, –20 and –20°C, respectively. Box Jellyfish Antivenom was obtained as ampoules containing 20 000 units in 2.5 mL. Chemicals were prepared in MilliQ water and stored as stock solutions at 4°C.

Statistical methods

Data are presented as the mean±SEM of n experiments. Responses were compared within (and between, where appropriate) isolated tissue groups by repeated-measures ANOVA with Greenhouse–Geisser correction for correlation,[29] calculated by means of the statistical program SuperANOVA™ 1.11 for Macintosh (Abacus Concepts, Berkeley, CA, USA). Basal forces in human isolated heart tissues were compared between groups by one-way ANOVA and experiments in the presence of atropine were analysed using paired Student's t-test. The effects of vehicle and CVE administration on cardiovascular parameters were compared within piglets by paired Student's t-test. Piglet plasma catecholamine levels were compared by repeated-measures ANOVA with Greenhouse–Geisser correction for correlation (with a post hoc test for pairwise comparisons where appropriate) and creatine phosphokinase levels before and after CVE administration were compared by paired Student's t-test. $P < 0.05$ was accepted as statistically significant.

RESULTS

Jellyfish morphology and venom yield

The overall low-power microscopic appearance of a typical *C. barnesi* jellyfish is shown in Fig. 1a (photographed using a Zeiss Optical System SV-11, Jena, Germany). A close-up of a tentacle showing the characteristic 'sleeve'-shaped bands of nematocysts is shown in Fig. 1b, with the morphology of a discharged nematocyst presented at higher magnification (Fig. 1c). The protein yield of a typical batch of 30 pooled *C. barnesi* jellyfish was 1.2 mg prepared in 3 mL PBS.

In vitro analysis

Right atria

In rat isolated spontaneously beating right atria, pretreatment with atropine (1 μmol/L) did not affect the basal rate (baselines in the control ($n = 8$) and TTX ($n = 3$) groups being 254 ± 23 and 255 ± 31 b.p.m., respectively). *Carukia barnesi* CVE (0.1–10 μg/mL) caused a concentration-dependent tachycardia ($P < 0.0001$, repeated-measures ANOVA; $n = 8$; Fig. 2a). This response was almost completely prevented by pretreatment with the Na^+ channel blocker TTX (0.1 μmol/L; $P = 0.047$ compared with the control group, repeated-measures ANOVA; Fig. 2a). Tetrodotoxin treatment itself did not alter baseline atrial rate ($P > 0.05$, paired t-test). Increases in atrial rate with the highest concentration of CVE (10 μg/mL) were 89 ± 11 and 24 ± 17 b.p.m. in control ($n = 8$) and TTX-treated ($n = 3$)

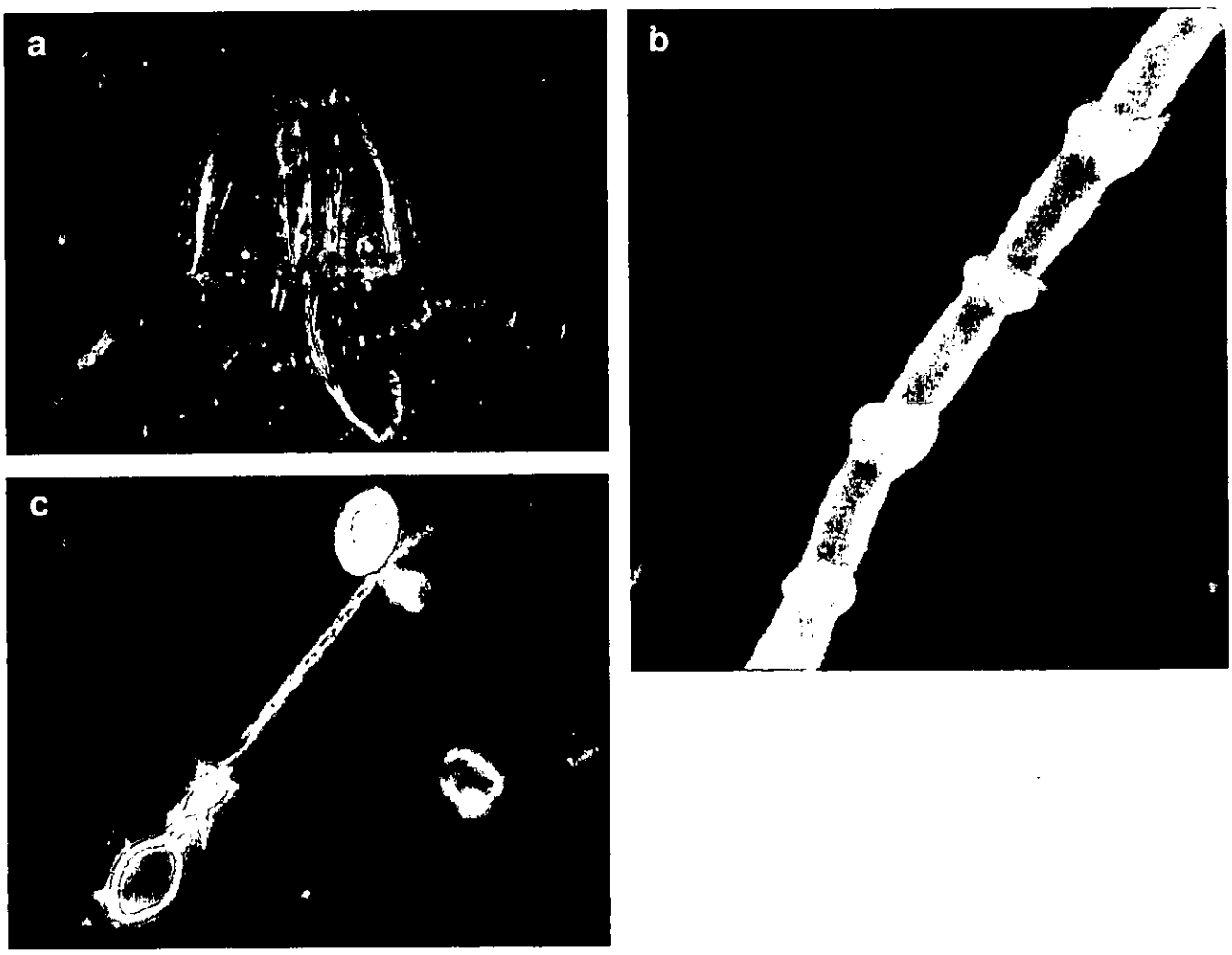

Fig. 1 (a) Photograph of a *Carukia barnesi* jellyfish showing the characteristic pyramidal-shaped medusa or bell (1 cm diameter at base), the lack of gastric cirri and the four retracted tentacles. Original magnification ×2.4. (b) A close-up of the characteristic 'sleeve' bands of nematocysts along the length of the tentacles. Original magnification ×10. (c) An example of the discharged tentacular nematocysts showing the ellipsoid nematocyte with shaft and spines (the nematocyst is approximately 25 × 18 μm wide). Original magnification ×40.

tissues, respectively. In separate rat atria (in the presence of 1 μmol/L atropine; $n = 3$), the N-type voltage-operated calcium channel blocker ω-conotoxin GVIA (0.1 μmol/L), also completely prevented any tachycardia in response to *C. barnesi* CVE (10 μg/mL; data not shown). In other atria, in the presence of 1 μmol/L propranolol, *C. barnesi* CVE caused a concentration-dependent bradycardia, with a fall from the baseline value of 260 ± 8 b.p.m. to 229 ± 5 b.p.m. with 3 μg/mL CVE ($P < 0.05$, repeated-measures ANOVA; $n = 6$; data not shown). Propranolol pretreatment did not alter baseline atrial rate. Like the tachycardia, this bradycardia was reversed by TTX (0.1 μmol/L; $n = 4$; data not shown).

In guinea-pig isolated right atria, atropine (1 μmol/L) pretreatment did not affect basal rate (148 ± 23 and 109 ± 15 b.p.m. in the control ($n = 4$) and TTX-treated ($n = 3$) groups, respectively). *Carukia barnesi* CVE appeared slightly more potent and efficacious than in rat atria; 3 μg/mL *C. barnesi* CVE caused a tachycardia of 130 ± 14 b.p.m. ($n = 4$; $P < 0.0001$, repeated-measures ANOVA; Fig. 2b). Pretreatment with 0.1 μmol/L TTX also essentially prevented the CVE-induced tachycardia ($P = 0.018$ compared with the control group, repeated-measures ANOVA).

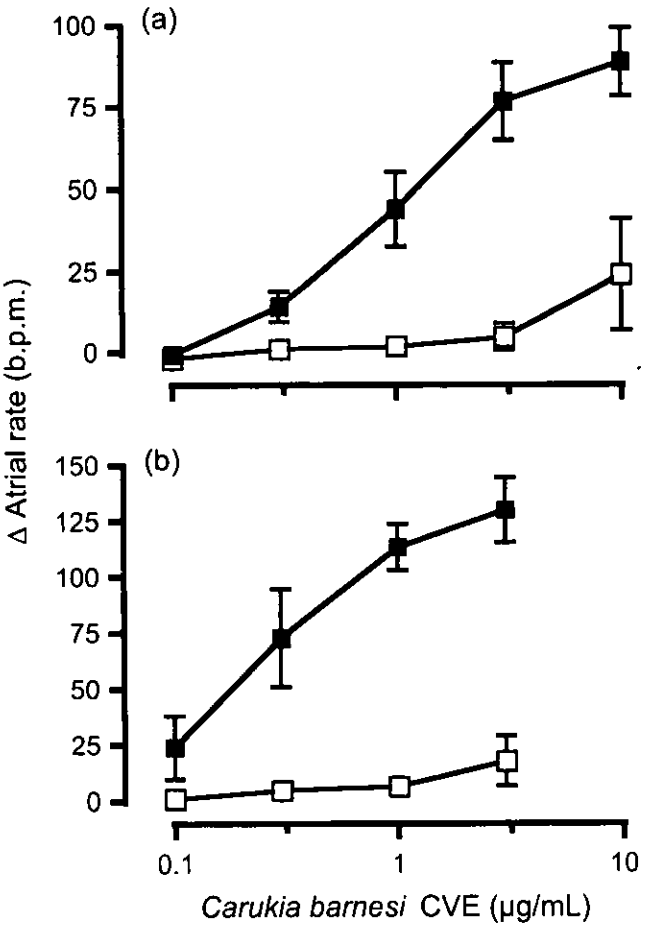

Fig. 2 Concentration–tachycardia response curves (expressed as change (Δ) in rate in b.p.m.) to *Carukia barnesi* crude venom extract (CVE) in (a) rat and (b) guinea-pig isolated spontaneously beating right atria in the presence of 1 μmol/L atropine. Responses to CVE are shown in control atria (■) from rats ($n = 8$) and guinea-pigs ($n = 4$) or in separate atria pretreated with 0.1 μmol/L tetrodotoxin (□) from rats ($n = 3$) and guinea-pigs ($n = 3$). Data are the mean±SEM (where not visible, the error is within the symbol).

Tetrodotoxin treatment itself did not affect the baseline rate ($P > 0.05$, paired t-test).

Left atria

In electrically driven (1 Hz) left atria pretreated with 1 μmol/L atropine, *C. barnesi* CVE elicited concentration-dependent positive inotropic responses of similar magnitude in rat and guinea-pig tissues, where 3 μg/mL *C. barnesi* CVE caused a doubling of the resting force of contraction ($P < 0.05$, repeated-measures ANOVA; $n = 3$; Fig. 3). Baseline atrial force was 0.39 ± 0.02 g in rat tissues ($n = 3$) and 0.19 ± 0.08 g in guinea-pig tissues ($n = 3$). In comparison, *C. barnesi* CVE (10 μg/mL) caused an increase in

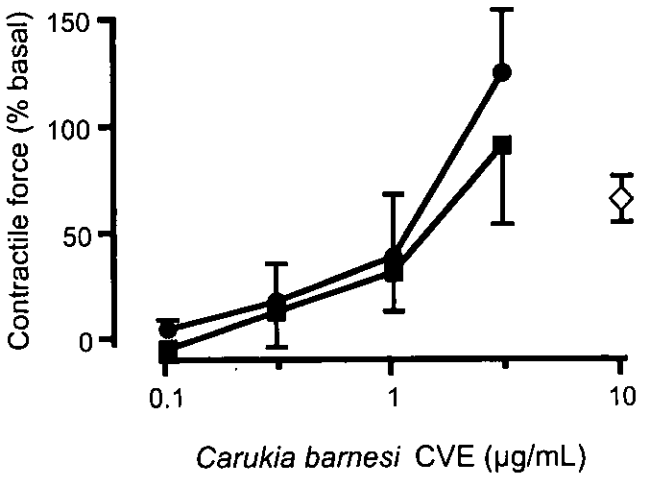

Fig. 3 *Carukia barnesi* crude venom extract (CVE) concentration–contraction curves in rat (■) and guinea-pig (●) isolated electrically driven left atria ($n = 3$ each). Data are the mean±SEM. For comparison, data from sixtrabeculae muscles from human right atrial appendage ($n = 5$ patients; ◇) are shown with 10 μg/mL CVE. Contractile force is expressed as percentage increase over basal force.

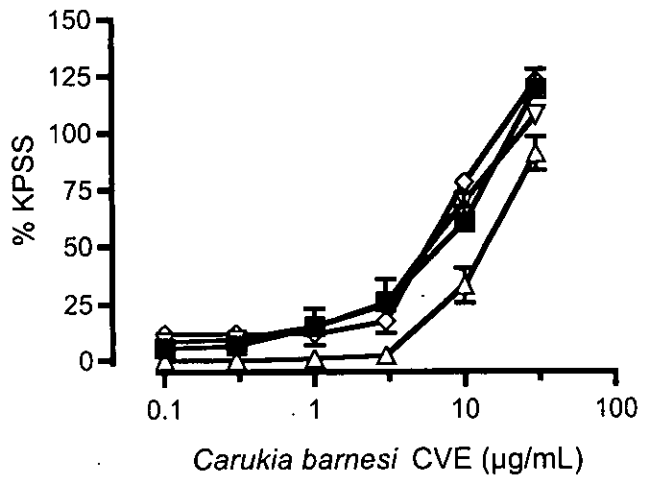

Fig. 4 *Carukia barnesi* crude venom extract (CVE) concentration–contraction curves in rat isolated mesenteric small arteries. Vasopressin (0.1 nmol/L) was used to cause a small contraction before applying one of the pretreatments as follows: (■), vehicle (H_2O; $n = 10$); (△), 0.1 μmol/L tetrodotoxin ($n = 4$); (▽), 0.3 μmol/L prazosin ($n = 4$); (◇), 0.1 μmol/L ω-conotoxin GVIA ($n = 4$). One curve to *C. barnesi* CVE (0.1–30 μg/mL) was then constructed in each artery. Contractions are the mean±SEM expressed as a percentage of the maximum contraction to potassium depolarizing solution (KPSS) in each artery (where not visible, the error is within the symbol).

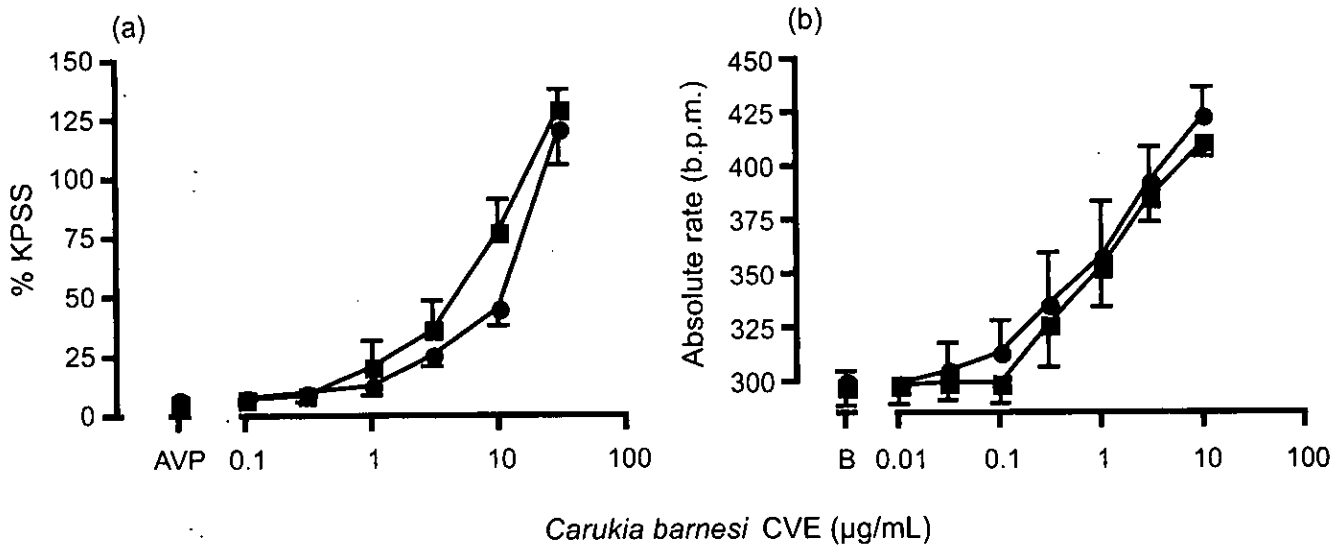

Fig. 5 Lack of effect of pre-incubation with *Chironex fleckeri* antivenom (92.6 units/mL final concentration) on *Carukia barnesi* crude venom extract (CVE)-induced (a) contractile responses in rat isolated small mesenteric arteries (expressed as a percentage of the maximum contraction to potassium depolarizing solution (KPSS)) and (b) tachycardic responses (expressed as absolute rate (b.p.m) in rat isolated right atria in the presence of 1 µmol/L atropine). (■), control (n = 7 and 3 for (a) and (b), respectively); (●), antivenom (n = 3 and 3 for (a) and (b), respectively). Data are the mean±SEM (where not visible, the error is within the symbol).

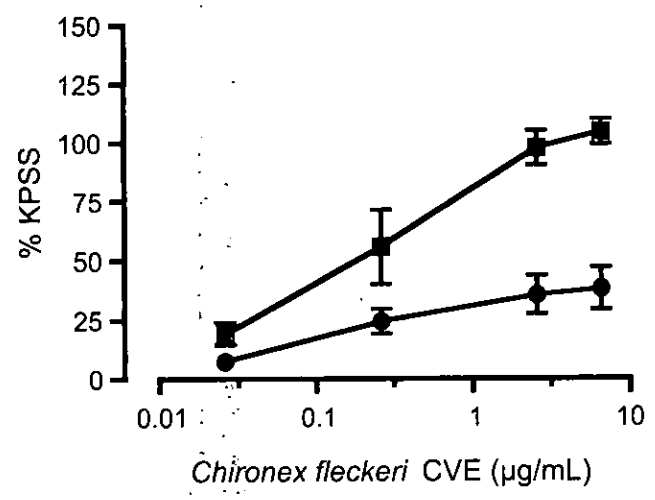

Fig. 6 Effect of *Chironex fleckeri* antivenom (92.6 units/mL; n = 6; ●) on contractile responses to *C. fleckeri* crude venom extract (CVE) in rat isolated small mesenteric arteries. (■), control responses in the absence of antivenom (n = 6). Contractions are presented as a percentage of the maximum contraction to potassium depolarizing solution (KPSS). Data are the mean±SEM (where not visible, the error is within the symbol).

basal contractile force of 67 ± 11% in human isolated trabeculae muscle (n = 6 tissues from five patients; Fig. 3). Limited stocks of *C. barnesi* CVE did not permit completion of full concentration–response curves and, therefore, it was not possible to compare its potency for rate and force changes.

Mesenteric artery

In rat mesenteric small artery, *C. barnesi* CVE (0.1–30 µg/mL) caused a sustained concentration-dependent contraction (P < 0.0001, repeated-measures ANOVA) that was not significantly altered by preincubation with TTX (0.1 µmol/L; n = 4), ω-conotoxin GVIA (0.1 µmol/L; n = 4) or prazosin (0.3 µmol/L; n = 4; P = 0.49 between groups, repeated-measures ANOVA; Fig. 4). The threshold for contraction in arteries (slightly precontracted with AVP) was 1–3 µg/mL CVE, concentrations similar to those that caused marked tachycardia and positive inotropic effects in isolated atria.

Chironex fleckeri antivenom

In separate rat mesenteric small arteries (precontracted 5–10% with AVP), concentrations of *C. barnesi* CVE were premixed for 30 min with box jellyfish (*C. fleckeri*) antivenom to give a final concentration of 92.6 units/mL before applying the CVE–antivenom mix to the artery chamber. The CVE–antivenom concentration–contraction response curve (n = 3) was not significantly different (P > 0.05, repeated-measures ANOVA) to the control CVE curve lacking antivenom (n = 7), indicating that the *C. fleckeri* antivenom afforded no protection from the *C. barnesi* CVE-induced contraction (Fig. 5a). Similarly, the tachycardia induced by *C. barnesi* CVE in rat right atria was unaffected by the *C. fleckeri* antivenom (P > 0.05, repeated-measures ANOVA; n = 3; Fig. 5b). Evidence that the *C. fleckeri* antivenom was effective at 92.6 units/mL is shown by its marked inhibition of the *C. fleckeri* CVE concentration–contraction curve in the rat mesenteric artery (P = 0.0009 between groups, repeated-measures ANOVA; n = 6 each; Fig. 6).

Human right atria

Carukia barnesi CVE (10 µg/mL) caused a biphasic response in human isolated right atrial trabeculae, with a tendency towards an initial rapid decrease in contractile force (–62 ± 12%; n = 7 tissues from five patients; P > 0.05, repeated-measures ANOVA), followed by an overall increase in force that was maintained (P < 0.05 Figs 7,8). However, the level of the contractile state was 'uneven' and was characterized by small fluctuations (e.g. Fig. 7). In the presence of atropine (1 µmol/L), CVE (10 µg/mL) caused a rapid increase in contractile force (P < 0.05, repeated-measures ANOVA

$n = 6$ tissues from five patients; Figs 7,8). The presence of atropine abolished the tendency of CVE to cause an initial fall in contractile force and also prevented the 'uneven' nature of the positive inotropic effect. In the presence of propranolol (0.2 μmol/L), CVE caused an initial decrease in contractile force that was now significant ($P < 0.05$, repeated-measures ANOVA), followed by a tendency towards an increase in force ($P > 0.05$ compared with basal values; $n = 7$ tissues from five patients; Figs 7,8). The 'uneven' characteristic of the level of contractile force was retained in the presence of propranolol. A higher concentration of propranolol (1 μmol/L) was added to the trabeculae following equilibration with CVE (10 μg/mL) and decreased the contractile force to its pre-CVE level after 18 min (Fig. 7b).

In vivo analysis

Cardiovascular parameters

In anaesthetized piglets, cardiovascular parameters before and 10 min after the administration of CVE (67 μg/kg, i.v. bolus) are summarized in Table 1. Significant increases were observed in mean arterial pressure, heart rate, pulmonary artery pressure, central venous pressure and cardiac output (all $P < 0.05$, paired Student's t-test; $n = 5$). No significant changes were observed in pulmonary artery occlusion pressure or in systemic or pulmonary vascular resistance ($P > 0.05$). The onset of systemic hypertension occurred within several minutes of CVE administration and persisted for 60–100 min. This was coincident with marked rises in

Table 1 Cardiovascular parameters at the peak of response to vehicle or *Carukia barnesi* crude venom extract in anaesthetized piglets ($n = 5$)

Cardiovascular parameter	Vehicle (180 μL/kg)	CVE (67 μg/kg)
Mean arterial pressure (mmHg)	79 ± 7	121 ± 14*
Heart rate (b.p.m.)	94 ± 14	143 ± 32*
Mean pulmonary artery pressure (mmHg)	18 ± 2	24 ± 4*
Central venous pressure (mmHg)	0 ± 1	2 ± 1*
Pulmonary artery occlusion pressure (mmHg)	8 ± 1	9 ± 1
Cardiac output (mL/min per kg)	160 ± 17	217 ± 26*
Systemic vascular resistance (mmHg/mL per min per kg)	0.51 ± 0.06	0.58 ± 0.09
Pulmonary vascular resistance (mmHg/mL per min per kg)	0.07 ± 0.01	0.08 ± 0.01

Values are the mean±SEM at the peak response to vehicle (phosphate-buffered saline) or crude venom extract (CVE; 10 min after i.v. administration). *$P < 0.05$ compared with vehicle (paired Student's t-test).

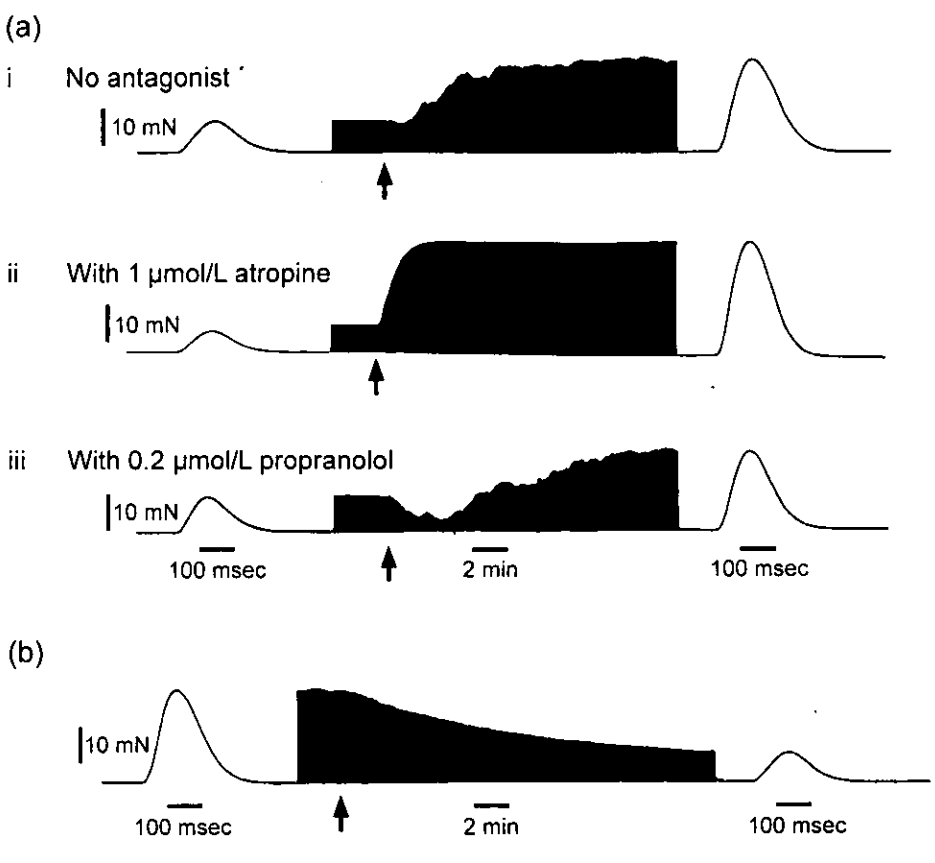

Fig. 7 (a) Cardiodepressant and stimulant effects of *Carukia barnesi* crude venom extract (CVE) on human isolated right atrial trabeculae. A representative experiment is shown on right atrial trabeculae from a 40-year-old male patient with three-vessel coronary artery disease treated with nicorandil only undergoing coronary artery bypass surgery. The computer scans of the original chart traces show the effects of 10 μg/mL *C. barnesi* CVE (i–iii; arrows) in the absence (i) or presence (ii) of 1 μmol/L atropine or in the presence of 0.2 μmol/L propranolol (iii). The CVE tended to cause a small, transient decrease in contractile force (not significant), followed by a sustained increase in contractile force (i). Atropine prevented the tendency towards an initial decrease in contractile force (ii). The CVE caused a transient fall in contractile force in the presence of propranolol (iii). Note in (i) and (iii), but not (ii), the 'uneven' characteristic of contractile force. (b) The trace is a continuation of (i) and shows complete reversal of the cardiostimulant effect of 10 μg/mL CVE by a higher concentration of propranolol (1 μmol/L; arrow).

serum catecholamines (Fig. 9). As an example of the effect of CVE, a record of systemic arterial pressure, pulmonary artery pressure and heart rate from a piglet immediately before and up to 90 min after administration is shown in Fig. 10.

Plasma catecholamines, creatine phosphokinase, blood gases and electrolytes

Increases were observed in plasma levels of noradrenaline ($P = 0.011$, repeated-measures ANOVA) and the noradrenaline methylation product adrenaline ($P = 0.036$) within 10 min of the i.v. injection of *C. barnesi* CVE ($n = 5$; Fig. 9). The levels of noradrenaline remained significantly elevated for several hours, although attenuation commenced within 1 h after CVE injection. Although adrenaline levels rose less markedly than those of noradrenaline, they attenuated far more quickly than those of noradrenaline.

In contrast, no significant changes were detected in the levels of DOPA, DHPG, dopamine or DOPAC over the same time period ($P > 0.05$, repeated-measures ANOVA; $n = 5$; data not shown). These catecholamines, or their metabolites, were not detected in the CVE itself (data not shown). The mean creatine phosphokinase level before CVE administration was 200 ± 53 IU/L (human range 30–200 IU/L) and 237 ± 109 IU/L at 60 min after CVE injection ($n = 3$). The creatine phosphokinase myocardial fraction levels at the same time points were 5.8 ± 1.4 μg/L (human range < 5 μg/L) and 6.8 ± 1.2 μg/L ($n = 3$).

Arterial blood gases and serum electrolytes taken at baseline and at the peak of increases in catecholamine levels (10 min after CVE injection) were not significantly different ($P > 0.05$, paired Student's *t*-test; $n = 5$; data not shown).

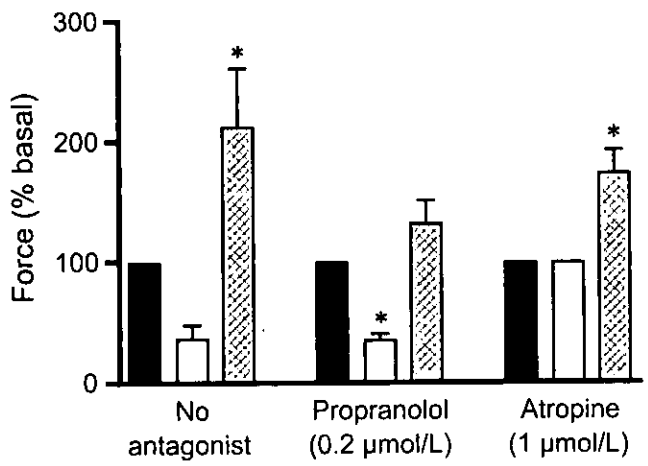

Fig. 8 Group data of the effects of 10 μg/mL *Carukia barnesi* crude venom extract (CVE) on contractile force (expressed as percentage of basal force) in human isolated right atrial trabeculae muscle in the absence or presence of propranolol (0.2 μmol/L) or atropine (1 μmol/L). (■), basal; (□), CVE minimum force (the minimum level of contractile force observed); (▨), CVE maximum force (the maximum level of contractile force observed). There was no difference in basal contractile force between the three groups (8.4 ± 1.7, 5.1 ± 1.6 and 7.8 ± 1.5 mN for no antagonist ($n = 7$), propranolol ($n = 7$) and atropine ($n = 6$ trabeculae), respectively). Data were obtained from $n = 6$–7 trabeculae from five patients and show the mean±SEM. *$P < 0.05$ compared with respective basal values (repeated-measures ANOVA with post hoc test).

DISCUSSION

The pharmacological basis of the Irukandji syndrome has long been the subject of speculation. Some clinical features of the syndrome resemble those of an adrenal medullary or catecholamine excess, such as seen in cases of phaeochromocytoma[1] and scorpion[30] or funnel-web spider envenomation.[31] Hitherto, it was reported that rats injected with *C. barnesi* venom exhibited systemic hypertension and raised serum catecholamine levels, although no data were provided.[1]

The present study demonstrates, for the first time, that venom extracted from *C. barnesi* jellyfish markedly increases serum catecholamines when infused into anaesthetized, ventilated piglets. This hypercatecholaminaemia was coincident with the onset of systemic and pulmonary hypertension, tachycardia and an increase in cardiac output. Therefore, the present data further support the concept that catecholamine excess is a key feature of this envenomation and suggest that a major mechanism of action of the venom is to modulate the activation of TTX-sensitive prejunctional neuronal Na^+ channels. Confirmation of this possibility would require electrophysiological studies. Certainly the *in vivo* pattern of catecholamine release suggests that this action of the venom is restricted to peripheral post-ganglionic sympathetic

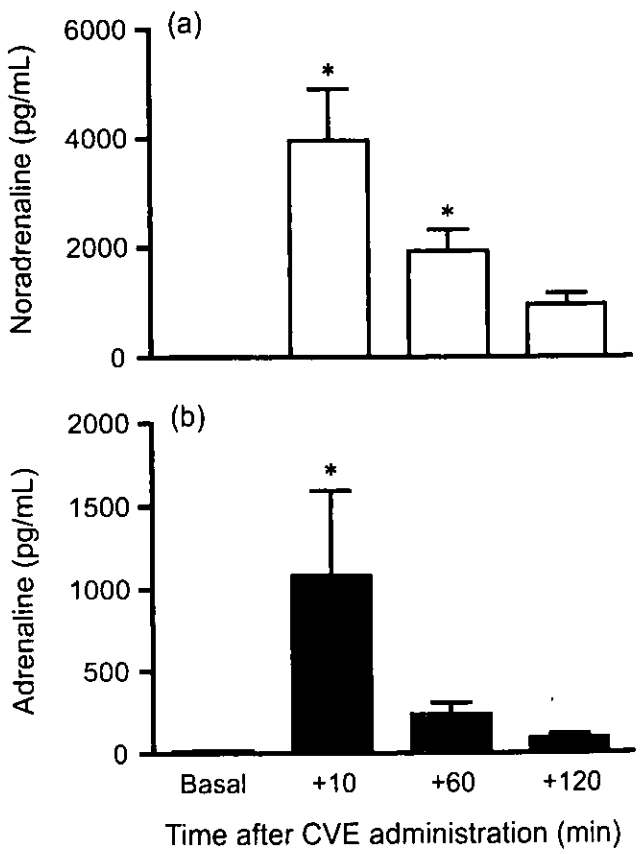

Fig. 9 Plasma concentrations of the catecholamines noradrenaline (a) and adrenaline (b) in anaesthetized piglets ($n = 5$). Levels are shown after i.v. administration of phophate-buffered saline (venom vehicle 180 μL/kg; basal) and 10, 60 and 120 min after the administration of *Carukia barnesi* crude venom extract (CVE; 67 μg/kg; +10, +60 and +120 min). Values are the mean±SEM. *$P < 0.05$ compared with respective basal values (repeated-measures ANOVA with post hoc test).

sites and possibly splanchnic nerve innervations and the adrenal medulla.

Following exposure to CVE, the contractile state of the heart appears to be determined by the net effect of cardiodepressant and -stimulant components, sensitive to atropine and propranolol, respectively. These are consistent with autonomic neuronal release of acetylcholine and noradrenaline. Although, clearly, the sympathetic effects dominate the clinical manifestations of this envenomation, the present study suggests that parasympathetic activation may also play a role in the dysautonomia manifest during the Irukandji syndrome.

It is of note that the similarities in the clinical features of scorpion and funnel-web spider envenomations reflect common sites of toxin action on the neuronal voltage-gated sodium channel (for a review, see Nicholson and Graudins[32]). Given the importance of this channel in nerve function, it will be invaluable to map the comparative properties and structure of any ion channel toxins in *C. barnesi* venom. However there also appears to be a TTX-insensitive vasoconstrictor effect of the *C. barnesi* venom extract. The mechanism underlying this second action remains to be determined. however, it is clear that both the TTX-sensitive and -insensitive actions are poorly, if at all, neutralized by the commercially available box jellyfish antivenom. This finding is consistent with the outcome of previously reported cases of Irukandji syndrome treated with this antivenom.[17,33] It also underlies the urgent need for further research into specific antagonists or antidotes to manage this potentially life-threatening condition. Whether the venom of other species of 'Irukandji' jellyfish is affected by the box jellyfish antivenom remains to be investigated. From the data so far, it seems probable that only the venom of the multitentacled or 'chirodropid' box jellyfish can be neutralized by this antivenom.[33,34]

The ability of CVE to alter the contractile state of the human heart has several implications. We would predict that patients at particular risk may include those with pre-existing cardiovascular pathologies, such as those associated with hypertension, ischaemic heart disease, arrhythmias and bleeding disorders. The treatment of cardiac symptoms and the hypertension seen in the Irukandji syndrome requires careful monitoring. Clearly, α- and β-adrenoceptor antagonists should be used with caution in conditions of catecholamine excess[35,36] and analgesia and supportive care remain paramount in the treatment of this envenomation.

Based on preliminary data from the present study,[21,33] it has been proposed that magnesium sulphate be used as a specific pharmacotherapy for the Irukandji syndrome.[37] Magnesium would decrease catecholamine release onto sympathetic nerve terminal receptors and lessen catecholamine-induced myocardial necrosis; it is a treatment used widely in some hyperadrenergic states.[37–39] Moreover, because the preliminary use of this drug in a therapeutic trial provided encouraging results, a multicentre randomized clinical trial and a dose-finding study are now underway to understand the role of magnesium in the treatment of this syndrome.[37,40] It is possible that this drug may obviate the need for an 'Irukandji' antivenom.[33] Note that recent studies of the cardiovascular effects of *C. fleckeri* venom reported that magnesium sulphate increased the efficacy of box jellyfish antivenom.[34] However, because this treatment is not without adverse effects,[40,41] its place in the management of Irukandji syndrome remains to be determined.

The present study has several limitations. First, although it is suspected that multiple jellyfish species are capable of causing the Irukandji syndrome,[1,8,14,15] the present study has focused on a single species, *C. barnesi*. Although it remains uncertain as to whether *C. barnesi* was actually responsible for either of the two fatalities

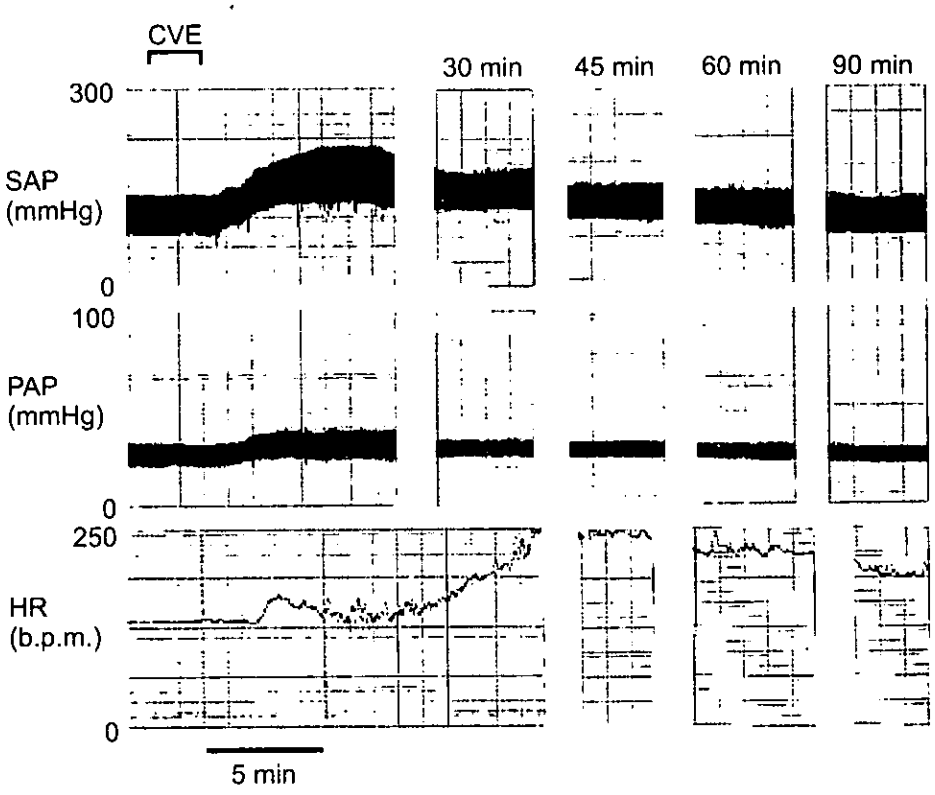

Fig. 10 Representative computer scan of a chart record before and after i.v. injection of Carukia barnesi crude venom extract (CVE; 67 mg/kg) in an anaesthetized, ventilated piglet. SAP, systemic arterial pressure; PAP, pulmonary artery pressure; HR, heart rate.

that occurred in 2002,[10,11] this jellyfish is clearly the major cause of the Irukandji syndrome in north Queensland.[3,10] Moreover, because this region has the greatest burden of the Irukandji syndrome in Australia,[10,42] *C. barnesi* deserves the greatest priority for study. Indeed, because no other species has yet been definitely identified as a cause of the syndrome, practically speaking no other jellyfish can yet be studied for an insight into the nature of this marine injury.

Second, the method used to prepare the venom extract may be subject to contamination by non-venom components. The optimal technique for the extraction of cubozoan venoms has long been the subject of debate.[43–45] Essentially three sources of venom extract have been used by various investigators: (i) 'milked' venom;[46,47] (ii) whole tentacle extracts;[44,48] and (iii) purified or enriched nematocyst preparations.[49,50] Although there has long been concern as to the clinical relevance of all components found in tentacle extracts,[43] 'milked' box jellyfish venom, in some ways the most natural venom preparation, has also been criticized for lacking lethal potency.[45] Newer nematocyst extracts[51] are contentious too for their potential for selective venom absorption.[52] However, the general conclusion appears to be that there is significant overlap in the venom components produced using the different methods[45] and that most pharmacological activity present in tentacle extracts reflects nematocyst contents.[44] The technique adopted here has been driven by the absolute lack of jellyfish material, concern regarding the thermolability of any venom components and their sensitivity to lyophilization. Given the congruency between the clinically observed syndrome,[8] the hypothesized mechanism of toxin action and other hypercatecholaminergic envenomations,[30,31,53] it is unlikely that the TTX-sensitive action described here is related to a non-nematocyst component. However, it is possible that the non-TTX-sensitive activity may represent a non-nematocyst, tentacular component. This is consistent with a report comparing the pharmacological activity of nematocyst versus tentacular extracts from *C. barnesi*.[54] Future studies should examine the various venom extraction techniques to clarify the structural origin of these two pharmacological activities.

Third, although most of these experiments have been undertaken using non-human tissues, the unequivocal confirmation of the relevance of those studies was seen when human right atria trabeculae were used. That the findings from the three animal tissues were completely consistent with those seen when human atrial tissue was used confirms their value. Given the greater variability and more limited accessibility of human tissue, we intend to continue to use such models in the future. However, we are currently studying the catecholamine levels in Irukandji syndrome patients to further confirm the clinical relevance of our findings. Such a study was recently undertaken in Venezuela. It directly related the extent of sympathetic nervous system activation, as measured indirectly through plasma noradrenaline levels in patients, with the severity of cardiovascular manifestations of *Tityus zulianus* scorpion envenomation.[53] The principal toxic components of such venoms are Na^+ channel neurotoxins.[55]

It has been hypothesized that a direct cardiotoxic action of *C. barnesi* and other 'Irukandji' venoms is responsible for the cardiac dysfunction sometimes seen in cases of Irukandji syndrome.[7,56] However, one does not even have to invoke a direct cardiotoxic action to explain the possibility of cardiac failure in this setting because excessive catecholamines can directly mediate myocardial necrosis.[57,58] The role of noradrenaline and β-adrenoceptor stimulation in the context of the aetiology of heart failure is now starting to be appreciated.[59,60]

The role of sympathetic nervous system activation in cardiotoxicity could be tested by pharmacologically denervating the animal prior to venom administration. This technique has been used previously to study the pathophysiology of funnel-web spider (*Atrax robustus*) envenomation;[61] this venom also contains a Na^+ channel modulator that causes excessive catecholamine release.[61,62] Moreover, the cardiac dysfunction and pulmonary oedema seen in that envenomation is prevented by prior α- and β-adrenoceptor and parasympathetic blockade.[61] Monkeys so treated survived an otherwise lethal dose of venom.

In summary, we conclude that *C. barnesi* venom may contain a sodium channel activator that stimulates the release of excessive catecholamines, explaining, at least in part, the clinical features of the Irukandji syndrome. Box jellyfish antivenom does not appear to be effective at neutralizing the action of this venom; however, a specific pharmacological antagonist for such a hyperadrenergic state may offer a new approach to treatment.

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