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THE BINDING OF SNAKE VENOM CARDIOTOXINS TO HEART CELL MEMBRANES

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Cobra venom cardiotoxins have the effect, inter alia, of causing systolic arrest of the heart. We have observed significant binding in vitro of ^{35}S-labelled cardiotoxins to mouse heart cell membranes. Part of the binding was saturable and could be displaced with homologous unlabelled cardiotoxins but not by neurotoxins or cardiotoxins inactivated by chemical modification. The specifically bound component represented more than 70% of total binding at saturation. Inclusion of Triton X-100 and NaCl in the phosphate-buffered incubation medium prevented nonspecific adsorption to centrifuge tube walls, and gave lower but more reproducible specific binding results, respectively. An apparent dissociation constant of $5 \cdot 10^{-7}$ M and a binding density of 500 pmol toxin/mg membrane protein were derived from the saturation isotherms.

Introduction

The venom of snakes belonging to the genera *Naja* and *Hemachatus* contain large amounts (up to 50% on a dry weight bases) of cardiotoxins, a group of low-molecular-weight ($M_r \approx 7000$) basic proteins [1–3]. Cardiotoxins affect various kinds of cell, both excitable and nonexcitable, causing irreversible depolarization and impairment of the structure and function of cell membranes [2,4–7].

The molecular basis of cardiotoxin action is still an unanswered question. Since the heart is known from pharmacological studies to be a target organ [8–12], the binding of cardiotoxin to heart cell membranes was investigated and the results are reported below. Labelled cardiotoxins isolated from the venom of banded Egyptian cobras (*Naja haje annulifera*) infused with radioactive amino acid precursors [13] were used. Analysis of the results according to the criteria for ligand-receptor interactions [14] revealed that cardiotoxins bind in a specific manner to heart cell membranes.

Materials and Methods

^{35}S- and ^{3}H-labelled cardiotoxins $V^{II}1$ and $V^{II}2$ were purified from radioactive venom obtained from captive banded Egyptian cobras (*Naja haje annulifera*) as previously described [13,15], except that isotopes were infused with the aid of Alzet miniature osmotic pumps (Model 2002, Alza Corp., Palo Alto) implanted intraperitoneally in the snake. [N,N^1-Dimethyllysyl]cardiotoxin $V^{II}4$ of *Naja mossambica mossambica* was prepared according to the method of Means and Feeney [16]. Mercurated cardiotoxin $V^{II}1$ of *Naja mossambica mossambica*, in which the four disulphides had been converted to S-Hg-S-bonds, was prepared according to Ref. 16. Methionine-sulphoxidized cardiotoxin $V^{II}1$ [18], neurotoxin d [19] and the cardiotoxin homologue $V^{II}3$ [20] of *Naja melanoleuca*, as well as bis[3-nitrotyrosyl-Tyr^{25}, Tyr^{51}]-

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Abbreviations: QNB, quinuclidinyl benzilate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulphate.

cardiotoxin $V^{II}1$ [21] of *Naja haje annulifera*, were gifts from Dr. F. Carlsson. L-[*benzylic-4,4'-*^{3}H]Quinuclidinyl benzilate (QNB; spec. act. 33.1 Ci/mmol) was obtained from New England Nuclear, Boston. Instagel II was a product of Packard Instruments. Atropine sulphate was supplied by Lennon Laboratories, Johannesburg. Bovine serum albumin (fraction V, fatty acid free) and porcine trypsin (three times crystallized, 4500 NFU/mg) were obtained from Miles Laboratories. Sigma Chemical Company supplied Triton X-100. All other reagents were of analytical quality and were supplied by Merck. Polyethylene centrifuge tubes (400 μl total volume) were purchased from Beckman Instruments, and polyethylene scintillation vial inserts were obtained from Weil Organisation, Johannesburg.

Membrane preparations. Fresh cardiac and brain cell membranes of male white mice (20–25 g body weight) were prepared for each experiment according to the method of Williams et al. [21], using 0.05 M phosphate (pH 7.4) as a buffer. Membrane pellets were suspended in buffer to 1 mg membrane protein/ml and kept on ice. Protein was determined by the modified Lowry method [23] with bovine serum albumin as a standard.

Pretreatment of membranes. Trypsinisation of the membrane preparation was carried out with trypsin (1 : 10 with respect to membrane protein) in 1 mM $CaCl_2$/0.05 M Tris-HCl (pH 8.0) for 60 min at 30°C. Membranes were subsequently pelleted at $10\,000 \times g$ for 2 min and washed twice with buffer to remove trypsin. Controls were run without trypsin.

Membrane thiol groups were reacted with 5,5'-dithiobis(2-nitrobenzoic acid). To a total of 2 mg membrane protein in 4 ml reaction buffer (0.1 M Tris/0.8 mM EDTA/35 mM sucrose (pH 7.8)) was added 16 μmol of DTNB in 400 μl of 0.05 M $NaKPO_4$ (pH 7.4) and the mixture was incubated for 10 min at 30°C. Excess DTNB was removed by decanting the supernatant after centrifugation (1 min at $10\,000 \times g$) and resuspending the pellet in 0.05 M phosphate (pH 7.4). The washing procedure was repeated twice. The number of membrane thiol groups that had been modified by DTNB was determined from absorbances at 412 nm after reacting treated membranes with dithiothreitol according to the method of Ellman [24], using a molar extinction coefficient of $\varepsilon_{412\,nm} = 1.36 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for liberated thionitrobenzoate anion.

The protease inhibitor phenylmethanesulphonyl fluoride (PMSF) was added to buffers during membrane preparation and binding experiments at a final concentration of 0.1 mM in 0.03% isopropanol, where appropriate.

Binding assays. Binding was determined by the miniature ultracentrifugation equilibrium method of Albers and Krishnan [25]. After incubation, membranes containing bound radioactive ligand were spun down within 60 s, an aliquot of the supernatant was removed and counted separately from the remaining pellet and its associated supernatant. The amount of bound ligand was calculated from the relationship

$$B = P - (F \cdot S)$$

where B, total dpm of bound ligand; P, dpm in pellet plus remaining supernatant; F, volume fraction = volume of remaining supernatant/volume of supernatant aliquot, and S, dpm in the supernatant aliquot. The volume occupied by the membrane pellet was sufficiently small to be neglected.

In a typical experiment, 100 pmol ^{35}S-labelled cardiotoxin was incubated with agitation for 30 min at 30°C with 50 μg membrane protein in a total volume of 200 μl incubation medium (50 mM phosphate/100 mM NaCl/0.01% Triton X-100) in capped polyethylene centrifuge tubes. Membranes were added last, after preincubation of reaction mixtures for 5 min at 30°C. After incubation, the membranes were immediately spun down in an Eppendorf microcentrifuge at $10\,000 \times g$ for 1 min. 100 μl of the supernatant (= supernatant aliquot, S) was transferred into a polyethylene vial insert, containing 50 μl of 20% SDS. The pellet left in the centrifuge tube with the remaining supernatant was resuspended and solubilized by the addition of 50 μl of 20% SDS, vortexing and incubation for 10 min at 80°C. Tube and contents were transferred into a vial insert (= pellet fraction, P). Radioactivities were determined to within the 95% confidence limit in 5 ml of Instagel scintillation fluid with a Packard liquid scintillation counter (Model 2660 or 3885).

All experimental points were determined in triplicate. Recoveries were monitored by compar-

ing the total radioactivity in the P and S fractions to the known amount initially added to the centrifuge tube. Nonspecific binding was determined in the presence of 100-fold excess of unlabelled cardiotoxin, and specific binding was taken as the difference between total and nonspecific binding. Binding data were further analysed as described by Fields et al. [26].

Results

Incubation conditions

Table Ia shows that there was appreciable nonspecific binding (approx. 35% of the added radioactivity) of cardiotoxin to the walls of the small polyethylene centrifuge tubes if incubations were done in the absence of membranes in 0.05 M phosphate buffer. Similar results were obtained with glass or polypropylene tubes. The adsorption effect could be abolished by inclusion of the nonionic detergent, Triton X-100, at a submicellar concentration of 0.01% (v/v) in the incubation medium. In a control experiment, this level of Triton X-100 did not affect the binding characteristics of the muscarinic antagonist ([^{3}H]QNB) to a brain cell membrane preparation. The observed apparent dissociation constant for QNB ($K_{50} = 1.3 \cdot 10^{-10}$ M) and the muscarinic receptor concentration at saturation ($R_m = 1.13$ pmol/mg membrane protein) were in agreement with literature values ($K_{50} = 1.6 \cdot 10^{-10}$ M; $R_m = 1.95$ pmol/mg membrane protein) established in the absence of Triton X-100 [27].

The effect of ionic strength of the incubation medium on the binding of cardiotoxin to heart cell membranes is clearly illustrated by the results in Table Ib. Total binding was 80% higher when 0.1 M NaCl was omitted from the phosphate buffer. Both the specific and nonspecific components of binding were increased in the low-ionic-strength phosphate buffer (by 100 and 43%, respectively). Reproducible recoveries (100 ± 6%) were obtained over a 20-fold concentration range of radioactive cardiotoxin when incubations were done in the Triton/saline/phosphate medium. The presence of Ca^{2+} at up to 2 mM concentration had no significant effect on binding. Routine binding assays were subsequently done in 0.05 M phosphate/0.1 M NaCl/0.01% Triton X-100 (pH 7.4).

TABLE I

THE EFFECTS OF TRITON X-100 AND NaCl ON THE BINDING OF LABELLED CARDIOTOXIN TO POLYETHYLENE OR HEART CELL MEMBRANES

(a) 300 pmol of ^{3}H-labelled cardiotoxin (3000 dpm) were incubated (30 min at 30°C) in polyethylene centrifuge tubes with 200 μl buffer (with or without Triton X-100) and the radioactivities recovered in the upper and lower halves of the solution were compared as decribed under Methods. Control experiments verified that the low-molecular-weight toxin was not differentially sedimented at 10000 × *g*. (b) 100 pmol of ^{35}S-labelled cardiotoxin (3500 dpm) and 65 μg membrane protein were incubated in the presence or absence of 100-fold excess of unlabelled cardiotoxin (see Methods).

Incubation medium	dpm bound		
	Total	Nonspecific	Specific
(a) Polyethylene			
Buffer (0.05 M $NaKPO_4$ (pH 7.4))	1070	1070	–
Buffer + 0.01% Triton X-100	0	0	–
(b) Heart cell membranes			
Buffer + Triton X-100	590	150	440
Buffer + Triton X-100 + 0.1 M NaCl	325	105	220

Specific binding at a fixed level of 100 pmol of cardiotoxin was found to be linear with membrane protein concentration over the range of 5–80 μg protein/200 μl reaction volume (Fig. 1).

Determination of binding constants

Binding to the heart cell membrane preparations was measured as a function of the concentra-

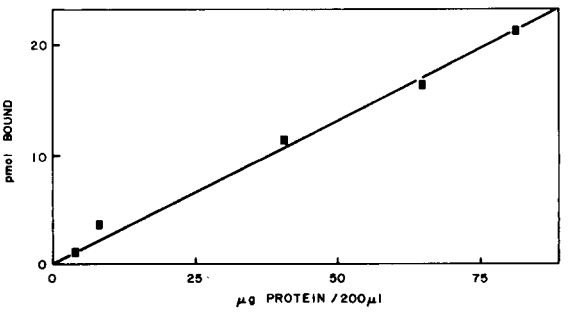

Fig. 1. Specific binding of ^{35}S-labelled cardiotoxin as a function of heart cell membrane concentration. The specific binding of 100 pmol ^{35}S-labelled cardiotoxin $V^{II}1$ to increasing amounts of membrane protein was determined as described in Methods.

tion of added ^{35}S-labelled cardiotoxin in the presence and absence of 100-fold excess of homologous unlabelled cardiotoxin. One component of toxin binding was reversible and saturable, displaying the rectangular hyperbolic concentration dependence characteristic of specific binding (Fig. 2). Nonspecific (i.e., non-displaceable) binding increased linearly under the experimental conditions employed. However, it comprised less than 25% of total binding at the stage when the specific component was already saturated. The equilibrium value for specifically bound toxin remained constant over incubation periods from 30 to 180 min, indicating that the binding interaction at 30°C was quite stable.

From a series of four independent experiments the mean value observed for half-maximal saturation (K_{50}) was $5.4 \cdot 10^{-7}$ M, whereas a maximum (R_M) of 524 (± 33) pmol ^{35}S-labelled cardiotoxin was bound per mg membrane protein at saturation. The apparent dissociation constant was also determined by displacing bound ^{35}S-labelled cardiotoxin with increasing amounts of homologous unlabelled cardiotoxin (Fig. 3). Half-maximal displacement was achieved at $5.8 \cdot 10^{-7}$ M of unlabelled cardiotoxin, which agrees with the value of the apparent dissociation constant obtained from the saturation isotherm.

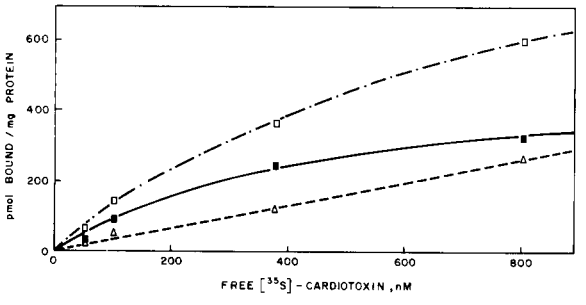

Fig. 2. Binding of ^{35}S-labelled cardiotoxin to heart cell membranes as a function of cardiotoxin concentration. Increasing amounts of ^{35}S-labelled cardiotoxin V^{II}1 of *Naja haje annulifera* (4–200 pmol) were incubated with heart cell membranes (65 μg/200 μl) in the presence and absence of 100-fold excess of unlabelled toxin. Each point represents the mean value of nine or more determinations with S.E. $\leq 6\%$. R_M = 525 pmol/mg protein; K_{50} = 466 nM. ■———■, specific; △— — —△, nonspecific; □— · —□, total binding.

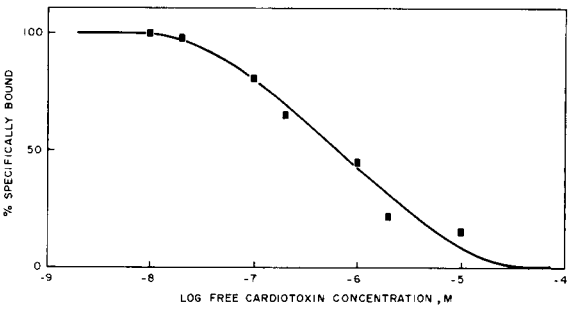

Fig. 3. Displacement of specifically bound ^{35}S-labelled cardiotoxin by unlabelled toxin. The specific binding of a constant amount (80 pmol) of ^{35}S-labelled cardiotoxin V^{II}1 to heart cell membranes (76 μg/200 μl) was determined in the presence of increasing amounts (2 pmol to 2 nmol) of homologous unlabelled cardiotoxin. $K_{50} = 5.8 \cdot 10^{-7}$ M.

Displacement with structural analogues

The ability of a number of structural analogues of cardiotoxin to displace specifically bound cardiotoxin is compared in Table II. Chemical modification or sequence variations which adversely affect cardiotoxicity also diminished displacement (competition) capacity. Neurotoxin d, for example, has an analogous sequence but no cardiotoxicity and cannot displace specifically bound cardiotoxin from heart cell membranes. Sulphoxidation of the methionines of cardiotoxin V^{II}1 from *N. melanoleuca* abolishes its toxicity [18] and lowered its ability to compete with native toxin for binding sites to an insignificant level. Similarly, *N*-methylated or *S*-mercurated cardiotoxins, which were no longer lethal to mice, even at high doses, were weak competitors for binding. By contrast, nitration had only a moderate effect on toxicity and did not seriously interfere with displacement capacity.

Chemical nature of the binding substance

The protease inhibitor, PMSF, had no effect on specific binding and pretreatment of membranes with trypsin resulted in a 30% increase in specific binding, indicating that the binding substance was not adversely affected by endogenous protease or exogenous trypsin. In fact, it seems as if new binding sites were exposed by trypsin.

Modification of thiol groups with DTNB caused a 35% decrease in specific binding and on average,

TABLE II

DISPLACEMENT OF SPECIFICALLY BOUND ^{35}S-LABELLED CARDIOTOXIN FROM HEART CELL MEMBRANES BY STRUCTURAL ANALOGUES

Displacement was with 100-fold molar excess. 200 pmol ^{35}S-labelled cardiotoxin V^{II}1 were used, as decribed in Methods.

Toxin	Cardiotoxicity	Displacement capacity (%)
Cardiotoxin V^{II}1 (*N. h. annulifera*)	+ +	100
Neurotoxin d (*N. melanoleuca*)	0	0
Mercurated (-S-Hg-S-) cardiotoxin V^{II}1 (*N.m. mossambica*)	0 [a]	0
[*N*,*N'*-Dimethyl]cardiotoxin V^{II}4 (*N. m. mossambica*)	0 [a]	25
Methionine-sulphoxidized cardiotoxin V^{II}1 (*N. melanoleuca*)	0 [a]	17
Dinitro-Tyr^{22},Tyr^{51}-cardiotoxin V^{II}1 (*N. melanoleuca*)	+	89

[a] Intravenous toxicity to mice over 24 h was tested at at least $5 \times LD_{50}$ doses.

about three thiol groups per molecule of membrane protein were modified, taking the mean molecular mass of a membrane protein as 100 000.

Discussion

The binding of ligands to non-receptor material often presents difficulties in the evaluation of binding results [14]. In the present investigation it was found that at the low concentrations employed, cardiotoxin adsorbed to a significant extent to polyethylene tubes (Table I). This can probably be ascribed to hydrophobic interactions between the polyethylene surface and certain regions of the cardiotoxin, which has a number of non-polar amino acid clusters in its structure [1]. These have been postulated [28,29] to contribute to the binding of cardiotoxins to cell membranes via interactions with the lipid bilayer. The inclusion of Triton X-100 at a level below the critical micelle concentration removed this extraneous component of nonspecific binding (Table I). The mild detergent did not affect binding to membranes, as judged from a comparison of binding constants obtained for the muscarinic antagonist QNB and literature values. Due precautions should therefore be taken in cardiotoxin binding studies in order to eliminate nonspecific adsorption, whether filtration or centrifugation methods and plactic or glass laboratory ware are used.

The binding of cardiotoxins to heart cell membranes was significantly affected by the electrolyte composition of the buffer medium. Lower and more reproducible binding results were obtained when the 0.05 M phosphate buffer was augmented with 0.1 M NaCl. It is not certain whether a buffer of low ionic strength promotes electrostatic binding of the highly basic toxin to oppositely charged membrane groups. Electrostatic interactions with negatively charged phospholipids have been invoked in the binding of cardiotoxins to axonal membranes and lipid vesicles, but were reported not to be much different for media of low or moderate ionic strength [30,31].

The observation that physiological concentrations of Ca^{2+} (not higher than 2 mM) had no effect on the binding of cardiotoxins has to be reconciled with firm evidence that Ca^{2+} is required for pharmacological effects [32]. However, only the initial event, viz. binding, could be studied with the heart cell membrane preparation and it is likely that Ca^{2+} is involved in the transduction of the binding interaction into a physiological response.

The observed apparent dissociation constant (approx. 10^{-7} M) suggests a moderate affinity of cardiotoxin for the membrane binding component, as compared to the dissociation constants of complexes of QNB ($K_d = 1.6 \cdot 10^{-10}$ M [26]) or peptide hormones ($K_d = 10^{-8}$ to 10^{-10} M, [33]) with their respective receptors. The binding strength of cardiotoxin lies in the range reported for effectors and agonists of the muscarinic or nicotinic cholinergic receptors, for example. In those studies, however, a subpopulation of receptors with much higher affinity ($K_d < 10^{-10}$ M) was also

shown to be present by the use of specific antagonists [26,34]. A similar distribution of high- and low-affinity receptors could not be identified in the present investigation, for a lack of known antagonists. The specific activity (10–20 mCi/mmol) and available amounts (for larger volume incubations) of labelled cardiotoxin were, furthermore, insufficient for this purpose. It is, nonetheless, noteworthy that the pharmacological concentration of cardiotoxin which causes half-maximal contraction of skeletal or heart muscle has been found to be about 10^{-7} M [9,35], i.e., of the same order of magnitude as its binding constant to heart cell membranes. While no direct physiological or pharmacological response of binding could be measured, it would appear that binding is related to subsequent events brought about by cardiotoxin. The good agreement between the values found for half-maximal displacement by unlabelled toxin and the dissociation constant derived from the saturation isotherms also indicated that ^{35}S-labelled cardiotoxin was biologically equivalent to native toxin as far as binding properties are concerned.

The saturation value of approx. 500 pmol toxin/mg membrane protein implies that there is a large number of binding sites (over 10^6) for cardiotoxin on a heart cell [36], which is even more than has been estimated to exist for the similarly sized epidermal growth factor on a fibroblast cell (40 000–1 000 000 [37]). Some of these binding sites may represent spare receptors, i.e., those not necessary for maximal response. The occurrence of spare receptors for insulin has, for example, been demonstrated on fat cells where maximal glucose oxidation (physiological response) was observed when only 2–3% of the total number of receptors was occupied [33]. Of course, all of the observed specific binding sites for cardiotoxin need not necessarily be of functional significance [14].

Chemical specificity of cardiotoxin binding was confirmed by displacement results with different structural analogues. The extent of competition by chemically modified toxins correlated fairly well with their reduced biological activity (Table II). The binding results with methionine sulphoxidized cardiotoxin lent further support to the notion that methionine residues are of critical importance in the mediation of the toxic effect of typical cardiotoxins [18]. The fact that a mercurated derivative could not displace any bound cardiotoxin and was also non-toxic (Table II) is ascribed to a grossly perturbed conformation, since its circular dichroism spectrum (not shown) resembled that of disordered cardiotoxin [38]. By contrast, *N*-methylated cardiotoxin appeared native as far as its conformation was concerned, but the rather mild modification of some of its amino groups caused a significant decrease in displacement capacity as well as a loss in toxicity. These results demonstrated that binding, like toxicity, is dependent not only on the overall conformational features of a cardiotoxin but also on the discrete properties of certain residues, such as their hydrophobicity and charge.

The results on the nature of the binding component in the heart cell membrane can only be considered as preliminary. The increase in specific binding after trypsin treatment could indicate either interconversion of existing or exposure of new binding components, as had been postulated for α-adrenergic receptors of liver plasma membranes [39]. The results do not allow a distinction to be made as to the chemical nature of the binding component, be it protein [40], phospholipid [30], or both. The possible involvement of a membrane protein is suggested by the marked decrease of almost 40% in specific binding after prior modification of membrane thiol groups. A toxin disulphide-membrane sulphydryl exchange reaction was proposed several years ago [41] as a possible mode of cardiotoxin action and recent studies on insulin-receptor interaction [42] have also implicated such an exchange at the membrane surface as a mechanism for eliciting cellular responses.

In conclusion, the characteristics of binding of snake venom cardiotoxins to heart cell membranes are considered to satisfy the criteria for a specific interaction [14] reasonably well. Binding to cell membranes of a primary target organ was found to be saturable and displaceable, with the displacement efficacy of structural analogues decreasing in parallel with their cardiotoxicity. Although not unusually high, the binding constant derived from the saturation isotherm correlated with physiologically active toxin levels. The results are therefore relevant to an understanding of the mode of action of cardiotoxins.

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