UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA - DEPARTAMENTO DE BIOQUÍMICA

Daniela Gois Beghini

NEUTRALIZAÇÃO DE ATIVIDADES FARMACOLÓGICAS E ENZIMÁTICAS DE VENENOS CROTÁLICOS PERANTE ANTIVENENOS

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutor em Biologia Funcional e Molecular - Área de Bioquímica

Orientador: Prof. Dr Sérgio Marangoni
Co-orientador: Prof. Dr. Stephen Hyslop

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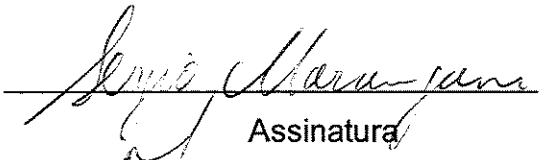

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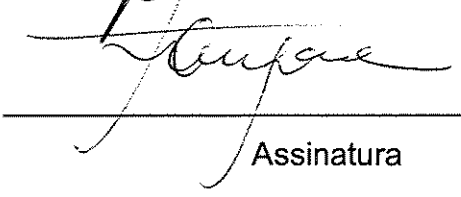

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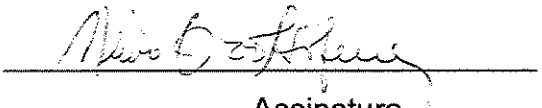

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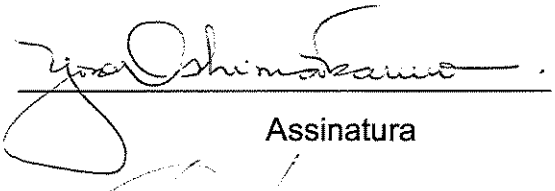

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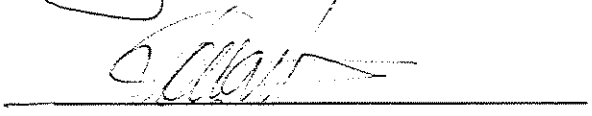

Assinatura

Prof. Dr. Paulo Pinto Joazeiro

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Prof. Dr. José Camillo Novello

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Resumo

Crotoxina, a principal neurotoxina do veneno das cascavéis *Crotalus durissus terrificus* e *Crotalus durissus cascavella,* é um complexo protéico que contém uma fosfolipase A_2 (PLA_2) básica e uma proteína ácida, crotapotina. O veneno e a crotoxina purificada do veneno de *C. d. cascavella* foram estudados quanto aos efeitos neurotóxico e miotóxico na preparação biventer cervicis de pintainho. O veneno e a crotoxina mostraram bloqueio neuromuscular nessa preparação em concentrações tão baixas quanto 0,2 μg/ml e 1 μg/ml. Quanto ao efeito miotóxico, a mionecrose foi mais marcante com altas concentrações de veneno e crotoxina (1, 5 e 25 μg/ml). Assim, o veneno de *C. d. cascavella* e sua crotoxina possuem um efeito neurotóxico preponderante e bastante potente nessa preparação, e uma ação miotóxica que foi observada somente em altas concentrações.

Para melhor entender o mecanismo de neutralização da crotoxina por anticorpos foram produzidos antissoros específicos contra a crotoxina e PLA_2 do veneno de *C. d. cascavella*. O título de anticorpos e a especificidade dos antisoros produzidos foram avaliados por ELISA e immunoblotting, respectivamente. A neutralização da atividade neurotóxica foi avaliada por intermédio de técnica miográfica nas preparações biventer cervicis de pintainho e nervo frênico diafragma de camundongo. A capacidade neutralizante dos antissoros na preparação de camundongo foi comparável à do soro comercial anticrotálico produzido contra o veneno de *C. d. terrificus*. O antissoro anti-crotoxina foi um pouco menos potente do que o antissoro anti-PLA_2 no processo de neutralização e isso confirma o papel central da PLA_2 no mecanismo de neurotoxicidade da crotoxina.

Neste trabalho, nós também avaliamos a habilidade do antissoro produzido em coelho contra a crotapotina do veneno *C. d. cascavella* em neutralizar a neurotoxicidade e miotoxicidade deste veneno e crotoxina, e inibir a atividade enzimática do complexo crotoxina

e da PLA_2. Este antissoro neutralizou parcialmente o bloqueio neuromuscular causado pelo veneno e crotoxina na preparação frênico nervo-diafragma de camundongo, sem prevenir o dano morfológico resultante ou inibir a atividade fosfolipásica. Em contraste, antissoros contra a PLA_2 e crotoxina neutralizaram o bloqueio neuromuscular e a atividade enzimática efetivamente. A neutralização parcial do bloqueio neuromuscular pelo anti-crotapotina sugere que este antissoro pode prevenir a interação da crotoxina com seu receptor causando dissociação do complexo crotoxina por se ligar a crotapotina.

Neste estudo, nós também examinamos a habilidade dos antissoros contra a crotoxina e PLA_2 em neutralizar a neurotoxicidade dos venenos de *Crotalus durissus terrificus* e *Bothrops jararacussu* e suas principais toxinas na preparação frênico nervo-diafragma de camundongo. Immunoblotting mostrou que o anti-crotoxina de *C. d. cascavella* reconheceu a crotoxina de *C. d. terrificus* e BthTX-I de *B. jararacussu*, enquanto que o antissoro contra a PLA_2 reconheceu a PLA_2 de *C. d. terrificus* e a BthTX-I de *B. jararacussu*. ELISA confirmou esta reatividade cruzada. Estes antissoros neutralizaram eficazmente o bloqueio neuromuscular causado pelo veneno e crotoxina de *C. d. terrificus* na preparação de camundongo em proporções semelhantes às usadas para neutralizar o veneno e a crotoxina de *C. d. cascavella*. Anti-crotoxina e anti-PLA_2 também neutralizaram eficientemente o bloqueio produzido pelo veneno e principal toxina de *Bothrops jararacussu*, porém doses mais altas dos antissoros foram necessárias para essa neutralização. Então, os resultados mostram reatividade cruzada entre esses venenos e suas toxinas principais e também mostra que o antissoro produzido contra PLA_2 eficazmente neutraliza a neurotoxicidade dos venenos de *C. d. terrificus* e *B. jararacussu* e suas toxinas PLA_2. Esses resultados, portanto, confirmam o importante papel da PLA_2 no mecanismo de neurotoxicidade dos venenos.

Abstract

Crotoxin, the main neurotoxin from venom of the rattlesnakes *Crotalus durissus terrificus* and *Crotalus durissus cascavella*, is a protein complex that contains a phospholipase A_2 (PLA_2) basic and an acid protein, crotapotin. The venom and the purified crotoxin from *C. d. cascavella* venom were studied with relationship to the neurotoxic and myotoxic effects in the chick biventer cervicis preparation. The venom and crotoxin showed neuromuscular blockade in this preparation at doses as low as 0,2 mg/ml and 1 mg/ml. With relationship to the myotoxic effect, the myonecrosis was stronger with higher doses of venom and crotoxin (1, 5 and 25 μg/ml). These results showed that the *C. d. cascavella* venom and its crotoxin possess a preponderant and quite potent neurotoxic effect in this preparation, and a myotoxic action that was observed only at higher doses.

To clarify the crotoxin neutralization mechanism by antibodies, specific anti-sera was produced against the crotoxin and PLA_2 from *C. d. cascavella* venom. The title of antibodies and specificity of the anti-sera raised in rabbits were tested by ELISA and immunoblotting, respectively. The neutralization of the neurotoxic activity was evaluated through myoghraphic technique in the chick biventer cervicis and mouse phrenic nerve diaphragm preparations. The neutralizing capacity of the anti-sera against crotoxin and PLA_2 in the mouse preparation was comparable to the commercial crotalic antiserum produced against the *C. d. terrificus* venom. The anti-crotoxin anti-sera was a little less potent than the anti-PLA_2 and this confirms the central role of PLA_2 in the mechanism of neurotoxicity of the crotoxin.

In this work, we also examined the ability of rabbit anti-serum raised against crotapotin purified from *Crotalus durissus cascavella* venom to neutralize the neurotoxicity and myotoxicity of this venom and crotoxin, and to inhibit the enzymatic activity of the crotoxin complex and PLA_2 alone. This anti-serum to crotapotin partially neutralized the neuromuscular

blockade caused by venom and crotoxin in electrically stimulated mouse phrenic nerve-diaphragm preparations, without preventing the resulting morphological damage or inhibiting the PLA_2 activity. In contrast, rabbit anti-sera to PLA_2 and crotoxin effectively neutralized the neuromuscular and PLA_2 activities. The partial neutralization of the neurotoxicity of crotoxin by the anti-serum to crotapotin suggested that this anti-serum may prevent the interaction of intact crotoxin with its recceptor by causing dissociation of the crotoxin complex through binding to crotapotin.

In this study, we also examined the ability of anti-sera raised against crotoxin and PLA_2 to neutralize the neurotoxicity of *Crotalus durissus terrificus* and *Bothrops jararacussu* venoms and their major toxins, crotoxin and bothropstoxin-I (BthTX-I), respectively, in mouse isolated phrenic nerve-diaphragm preparations. Immunoblotting showed that anti-serum to crotoxin from *C. d. cascavella* recognized crotoxin from *C. d. terrificus* and BthTX-I from *B. jararacussu*, while anti-serum to PLA_2 from *C. d. cascavella* recognized PLA_2 from *C. d. terrificus* and BthTX-I from *B. jararacussu*. ELISA corroborated this cross-reactivity. These anti-sera efficiently neutralized the neuromuscular blockade caused by venom and crotoxin from *C. d. terrificus* in mouse preparation in similar proportions used to neutralize the venom and crotoxin from *C. d. cascavella*. Anti-crotoxin and anti-PLA_2 anti-sera also efficiently neutralized this blockade produced by venom and main toxin of *Bothrops jararacussu*, bothropstoxin-I (BthTX-I), however higher doses of anti-sera was necessary for this neutralization. Therefore, the results show cross-reactivity between these venoms and its main toxins and also shown that anti-serum produced against PLA_2 efficiently neutralized the neurotoxicity of *C. d. terrificus* and *B. jararacussu* venoms and their PLA_2 toxins. These results confirms the important role of PLA_2 in the neurotoxicity mechanism of the venoms.

Introdução

INTRODUÇÃO

Os acidentes ofídicos têm reconhecida importância médica, em virtude do grande número de pessoas atingidas e pela própria gravidade que encerram, pois apresentam índices significantes de morbidade e mortalidade em muitos países. Acidentes ofídicos envolvendo espécies do gênero *Crotalus* são geralmente sérios e freqüentemente fatais na ausência de tratamento adequado e específico (BARRAVIERA et al.,1989). Apresenta o maior índice de letalidade (1,87%) devido à freqüência com que evolui para insuficiência renal aguda, que é a mais séria complicação do envenenamento crotálico humano (CUPO et al.,1988; MINISTÉRIO DA SAÚDE, 1998).

O diagnóstico clínico do envenenamento crotálico baseia-se, principalmente, na observação de manifestações sistêmicas decorrentes das ações neurotóxica, miotóxica e coagulante do veneno (ROSENFELD, 1971). A atividade neurotóxica é conseqüência do bloqueio na junção neuromuscular causando uma paralisia flácida, e caracterizam a face miastênica (face neurotóxica de Rosenfeld) evidenciadas por ptose palpebral uni ou bilateral, flacidez da musculatura da face, alteração do diâmetro pupilar, incapacidade de movimentação do globo ocular (oftalmoplegia), podendo existir dificuldade de acomodação (visão turva) e ou visão dupla (diplopia) (MINISTÉRIO DA SAÚDE, 1998).

A atividade miotóxica parece ser responsável pelas dores musculares generalizadas (mialgias) e por discreto edema no local da picada. A urina de cor avermelhada ou de tonalidade mais escura é a manifestação clínica mais evidente da intensidade da rabdomiólise, sendo a mioglobinúria responsável pela insuficiência renal aguda. Em conseqüência disso podem ocorrer grandes elevações séricas de creatinofosfoquinase e da desidrogenase láctica (AZEVEDO-MARQUES et. al., 1985).

As manifestações nos locais da picada nos envenenamentos por cascavéis são pouco importantes diferindo dos acidentes botrópico e laquético. A atividade coagulante de pequena

intensidade e a inexistência de atividade proteolítica faz com que, no local da picada, não haja alterações ou, quando existirem, sejam reduzidas a um discreto edema ao redor do ponto de inoculação do veneno (VITAL BRAZIL,1982 ; MINISTÉRIO DA SAÚDE, 1987). No caso da *Crotalus durissus cascavella* especificamente danos no local da picada têm sido reportados como sendo mínimos ou ausentes (AZEVEDO-MARQUES et al., 1985).

O tratamento dos envenenamentos é feito pela soroterapia, introduzido por CALMETTE (1894) usando soros antiofídicos. Desde o início do século, poucas modificações foram introduzidas na preparação dos soros antiofídicos. Uma das abordagens visando a melhora dessas preparações consiste no enriquecimento dos antivenenos comerciais com anticorpos dirigidos contra um determinado componente tóxico do veneno. Assim, RUSSEL et al. (1985) utilizaram anticorpos isolados por cromatografia de afinidade, para aumentar a capacidade de antivenenos comerciais nas ações anti-letal, anti-citolítica, anti-hemorrágica e anti-agregação de plaquetas. Em uma abordagem semelhante (OWNBY et al., 1983 a, b) sugeriram que anticorpos anti-miotoxinas têm uma ação neutralizante da atividade mionecrótica do veneno, superior à do antiveneno comercial. A adição de um soro anti-miotoxina ao antiveneno comercial, em iguais proporções, melhorou a atividade anti-miotóxica do mesmo, sem alterar a sua atividade anti-hemorrágica ou anti-letal (OWNBY et al., 1985). Existem ainda algumas evidências de que a imunização com apenas os componentes mais envolvidos na patologia do envenenamento possa gerar soros eficientes na neutralização do veneno. Desta forma, já foi demonstrado que a imunização de cavalos com a fração que possui atividade de fosfolipase A_2, poderia ser suficiente para produção de antisoro com capacidade de neutralizar a ação do veneno total de *C. d. terrificus* (HANASHIRO et al., 1978; SANTOS et al., 1988).

Sendo que os venenos são misturas complexas de componentes antigênicos diferentes, é esperado que anticorpos sejam produzidos em grande quantidade se os

animais forem imunizados somente com o principal componente tóxico do veneno. Diferentes autores reportaram que a imunização com uma única toxina produz consideravelmente mais alto título de anticorpos contra a toxina específica do que aqueles imunizados com várias toxinas ao mesmo tempo (RAMLAU et al., 1979, LIAU et al. , 1982). Especificamente no caso do veneno de *C. d. terrificus*, FREITAS et. al. (1990) mostraram um alto nível de proteção obtida em rato após a imunização com crotoxina, o componente neurotóxico do veneno.

A crotoxina do veneno de *C. d. terrificus* é representativa de um grande número de neurotoxinas présinápticas encontradas nos venenos de cascavéis. Um interesse considerável tem sido mostrado nessas toxinas a fim de conhecer detalhadamente seu mecanismo de ação e também melhor entender a liberação do neurotransmissor acetilcolina. HENDERSON E BIEBER (1986) sugeriram que a toxicidade elevada do veneno de algumas espécies do gênero *Crotalus* esteja associada à presença de antígenos semelhantes à crotoxina ou toxina Mojave. Anticorpos monoclonais e policlonais produzidos contra essas neurotoxinas têm sido uma ferramenta importante para que suas funções biológicas sejam mais bem entendidas (KAISER E MIDDLEBROK, 1988).

1. Crotoxina

Crotoxina foi primeiro isolado do veneno da cascavel Sul americana, *Crotalus durissus terrificus*, por SLOTTA E FRAENKEL-CONRAT em 1938. Por três décadas a crotoxina foi a única neurotoxina conhecida a ser isolada de veneno crotálico. Evidências acumuladas gradualmente sugeriram que outros venenos de cascavéis com baixos valores de DL_{50} continham uma toxina similar (HENDON E BIEBER, 1982). Estudos cromatográficos, elétroforéticos e biológicos demonstraram que isso era verdade para os venenos de *Crotalus scutulatus scutulatus* (Mojave rattlesnake) e *Crotalus viridis concolor* (AIRD & KAISER,

1985). A principal toxina desses três venenos é uma proteína heterodimérica composta de subunidades ácidas e básicas.

Em 1985, AIRD *et. al.* seqüenciaram as cadeias A e C da subunidade ácida, crotapotina da crotoxina e, em 1990, o mesmo grupo forneceu a sequência completa da cadeia B da subunidade ácida usando espectometria de massa (AIRD *et al*., 1990). A seqüência de aminoácidos da subunidade básica, fosfolipase A_2 (PLA_2), foi completada em 1986 por AIRD *et. al*.. A PLA_2 é uma única cadeia polipeptídica de 122 resíduos de aminoácidos e sua seqüência é homóloga a de outras PLA_2 de pâncreas e venenos de serpente (AIRD *et. al*.,1986). A crotapotina consiste de três cadeias polipeptídicas ligadas por sete pontes dissulfeto (BREITHAUPT *et al*., 1974), as seqüências de aminoácidos de suas três cadeias são homólogas a várias partes de uma PLA_2 sugerindo que a crotapotina é produzida por proteólise limitada de um precursor fosfolipase (AIRD *et al*., 1985)

A crotoxina é a principal proteína constituinte do veneno de *C. d. terrificus* e pertence a classe das β-neurotoxinas. As β-neurotoxinas são neurotoxinas présinápticas caracterizadas por apresentar atividade fosfolipásica (BON, 1997). Elas são consideradas potentes neurotoxinas as quais bloqueiam a transmissão neuromuscular, principalmente por modificação na liberação do neurotransmissor (HAWGOOD E BON, 1990). Além da ação présináptica principal, a maioria destas toxinas atua, também, em doses geralmente maiores, na membrana das fibras musculares, onde podem provocar diminuição ou perda da excitabilidade e mionecrose (GOPALAKRISHNAKONE E HAWGOOD, 1984)

A crotoxina é uma proteína heterogênea. Várias isoformas já foram isoladas do veneno de uma única serpente e resultam da associação de várias isoformas de suas duas subunidades (FAURE E BON, 1988). As várias isoformas de PLA_2 resultam da expressão de diferentes RNAs mensageiros (BOUCHIER *et al*., 1991). As isoformas de crotapotina derivam

de um único precursor, homólogo a uma PLA_2, por diferentes eventos pós-translacionais (BOUCHIER *et al*., 1991).

As duas subunidades da crotoxina atuam de maneira sinérgica. A PLA_2 sozinha é fracamente tóxica e pode bloquear a transmissão neuromuscular, embora altas doses sejam necessárias para este efeito. A PLA_2 pode ligar em várias membranas e a crotapotina comporta-se como um chaperone por prevenir a ligação da PLA_2, restringindo assim sua ligação a sítios presentes nas membranas sinápticas (BON *et al*., 1979). Ao interagir com membranas ou vesículas de fosfolipídios, o complexo crotoxina dissocia-se: a PLA_2 liga-se, enquanto a crotapotina é liberada (DELOT E BON, 1993). O tempo de dissociação da crotoxina na presença de membranas présinápticas foi estudada determinando a cinética da ligação da crotoxina radiomarcada na presença de crotapotina ou crotoxina. Esses estudos sugerem que a crotapotina é temporariamente envolvida na ligação da PLA_2 a seu receptor. A crotoxina ligaria ao receptor, formando, portanto um complexo ternário, depois do qual uma mudança conformacional ocorreria induzindo a dissociação da crotapotina (DELOT E BON, 1993).

Além de ter um efeito neurotóxico, a crotoxina é também miotóxica, afetando diretamente as células do músculo esquelético (GOPALAKRISHNAKONE *et al*.,1981). A toxicidade ao músculo esquelético foi demonstrada *in vivo* por GOPALAKRISHNAKONE *et al*. (1984).

2. Neutralização da crotoxina por anticorpos

Os venenos são misturas complexas de macromoléculas, com vários epítopos capazes de estimular muitos clones de anticorpos secretados por linfócitos. A maioria dos antivenenos comerciais existentes é de origem eqüina e constituídos por IgG intactas ou por

fragmentos $F(ab)_2$. Apenas 5 a 13% dos anticorpos, ou fragmentos destes, ligam-se especificamente aos componentes do veneno usados para imunizar o cavalo (SJÖSTROM *et al*., 1994). A imunização é realizada com venenos de um número limitado de espécies do mesmo gênero, ou de diferentes gêneros para aumentar o espectro de neutralização do antiveneno (RUSSEL, 1988).

A crotoxina do veneno de *C. d. terrificus* é representativa de um grande número de neurotoxinas pré-sinápticas encontradas nos venenos de cascavéis. Um interesse considerável tem sido mostrado nessas toxinas a fim de conhecer detalhadamente seu mecanismo de ação e também entender melhor a liberação do neurotransmissor acetilcolina. Anticorpos monoclonais e policlonais produzidos contra essas neurotoxinas têm sido uma ferramenta importante para que suas funções biológicas sejam mais bem entendidas (KAISER E MIDDLEBROK, 1988). Para o veneno de *C. d. terrificus* já foi demonstrado que o antisoro produzido contra a crotoxina neutraliza os maiores efeitos letais do veneno (FREITAS *et al*., 1990; OSHIMA-FRANCO *et al*., 1999).

Antivenenos produzidos em coelhos contra o veneno total e crotoxina de *C. d. terrificus* são melhores do que antivenenos eqüinos em neutralizar suas atividades neurotóxicas e miotóxicas (OSHIMA-FRANCO *et al*., 1999). Anticorpos policlonais também foram produzidos em coelhos contra as duas subunidades da crotoxina. Os métodos de ELISA e imunodifusão de Ouchterlony têm sido utilizados (KAISER *et al*, 1986) para determinar a eficácia relativa de antissoros contra a crotoxina e suas subunidades, em camundongos. Anticorpos anti-PLA_2 neutralizaram a potência letal da crotoxina e inibiram sua atividade enzimática (CHOUMET *et al*., 1989). KAISER e MIDDLEBROOK (1988) produziram anticorpos monoclonais contra a PLA_2 da crotoxina através de imunização em camundongos, e mostraram suas propriedades neutralizantes frente aos efeitos tóxicos da crotoxina. Um antissoro produzido em coelho contra a subunidade básica da crotoxina foi

capaz de neutralizar a atividade letal da crotoxina intacta e do veneno (que contém crotoxina) em camundongos (HANASHIRO *et al*., 1978). CHOUMET *et al*., (1998; 1992) produziram anticorpo monoclonal contra a crotapotina e mostraram a neutralização da toxicidade da crotoxina, entretanto esse anticorpo não inibiu sua atividade enzimática.

3. Miotoxinas

MEBS E OWNBY (1990) definem miotoxicidade como uma ação específica do veneno sobre o músculo esquelético, causado por substâncias denominadas miotoxinas, que exercem, portanto, uma ação direta e específica sobre o músculo esquelético, levando a degeneração e morte celular (mionecrose). As miotoxinas encontram-se amplamente distribuídas nos venenos de serpentes (MEBS *et al*., 1983).

A crotoxina e sua PLA_2 induzem necrose no músculo esquelético (GOPALAKRISHNAKONE *et al*, 1984) e pertencem ao grupo de miotoxinas fosfolipásicas neurotóxicas (MEBS E OWNBY,1990). O efeito miotóxico em sítios distantes do local da picada é uma observação comum em pacientes envenenados com veneno de *C. d. terrificus* (CUPO *et al*., 1988). O efeito miotóxico sistêmico desse veneno foi primeiro demonstrado por AZEVEDO-MARQUES *et al*., (1982). SALVINI *et. al*., (2001) mostrou que a crotoxina induz a uma injúria muscular sistêmica e seletiva.

Estudos de microscopia eletrônica mostram que a crotoxina, bem como outras toxinas pertencentes a este grupo, levam a uma rápida degeneração da fibra muscular, evidente num período de 30 minutos a 3 horas. As alterações são caracterizadas por hipercontração de sarcômeros e presença de lesões do tipo “delta”. Mitocôndrias também mostram sinais de danos, como intumescimento, cristas anormais, degeneração flocular e ruptura. Entre 12 e 24 horas após a administração da crotoxina, as fibras musculares encontram-se totalmente

destruídas com aparência amorfa e hialina e contém muitas células fagocitárias, enquanto que a lâmina basal e microcirculação são preservadas (MEBS E OWNBY, 1990).

Estudos morfológicos sugerem que a ação primária dessas toxinas seja a hidrólise de fosfolipídeos da membrana plasmática da célula muscular (HARRIS E MacDONELL, 1981; GOPALAKRISHNAKONE *et al.*, 1984). Entretanto, não há um consenso se a atividade enzimática dessas PLA_2 é ou não requerida para sua ação nas células musculares esqueléticas (MEBS E OWNBY, 1990).

4. Reatividade e Neutralização cruzada

No Brasil, o gênero *Bothrops* é responsável por 90% de todas as picadas de serpentes peçonhentas registradas (MINISTÉRIO DA SAÚDE DO BRASIL – FUNDAÇÃO NACIONAL DE SAÚDE, 1999). O envenenamento bothrópico é caracterizado por inflamação, edema e necrose no local da picada com extenso dano tecidual local e efeitos sistêmicos tais como hemorragias, desfibrinação e trombocitopenia, com falência renal como um complicador comum (GUTIÉRREZ E LOMONTE, 1989; MANDELBAUM et al., 1989; GUTIÉRREZ, 1990). Envenenamento por *C. d. terrificus* é caracterizado por uma síndrome neurotóxica, a qual pode levar à morte por paralisia respiratória (AZEVEDO-MARQUES et al., 1990) e miotoxicidade, a qual pode produzir falência renal aguda (AZEVEDO-MARQUES et al., 1985, 1987). Quando se analisa a composição do veneno de famílias diferentes, observa-se que alguns componentes são largamente distribuídos em várias espécies, e outros são restritos a poucas espécies.

Em 1903, já se discutia sobre a especificidade dos soros antiofídicos, pois os soros produzidos por Calmette não neutralizavam a ação dos venenos das serpentes brasileiras, e mesmo entre os venenos das serpentes encontradas na América do Sul, o soro anticrotálico

não era capaz de neutralizar a ação letal do veneno de *Bothrops jararaca* (BRAZIL, 1903). Entretanto, muitos experimentos também demonstravam a presença de antígenos comuns nos venenos ofídicos de diferentes famílias, gêneros ou espécies. Com o avanço das técnicas que pudessem demonstrar as reações cruzadas entre os venenos tais como: imunodifusão em gel de agarose (OUCHTERLONY, 1949), e mais recentemente ELISA (CHÁVEZ-OLÓRTEGUI et al., 1993), muitos trabalhos demonstraram a composição ideal de antígenos para a produção de antivenenos (GRASSET et al., 1956; THEAKSTON E REID, 1979), pois a presença ou não de componentes comuns no veneno de espécies do mesmo gênero faz com que o venenos de certas espécies sejam indispensáveis nas formulações antigênicas, enquanto outras se tornam redundantes. Assim, para se obter um antiveneno efetivo, é importante conhecer a composição antigênica dos venenos das serpentes (CAMEY et al., 2002). Na maioria dos casos, a espécie responsável pelo envenenamento é raramente identificada e há uma demora na administração do antiveneno. O antiveneno ideal deveria estar prontamente disponível para um médico administrá-lo imediatamente, e deve mostrar reação cruzada com um grande número de venenos, além de causar poucas reações alérgicas em humanos (SÁNCHEZ et al., 2003).

A neutralização de venenos de espécies diferentes daquelas usadas na imunização pode ocorrer devido à reatividade cruzada. Os venenos podem estar relacionados entre si farmacológica e ou estruturalmente, o que permite a conseqüente neutralização por antiveneno heterólogo (MÉNEZ, 1985).

Há muitos antivenenos diferentes produzidos no mundo (THEAKSTON & WARREL, 1991). Os diferentes laboratórios produtores de antivenenos adotam procedimentos diferentes tais como (1) o tipo de animal usado, (2) o veneno de serpente usado, (3) protocolos de imunização, (4) clivagem da molécula de IgG, e (5) procedimentos de purificação. Apesar dessas diferenças, a maioria dos antivenenos mostra reatividade cruzada

com venenos que não são usados no protocolo de imunização. Há muitas considerações importantes a serem feitas na produção de um antiveneno polivalente. Entretanto, a mais importante delas é começar com a combinação correta de venenos que melhor representa a área onde o antiveneno será usado (SÁNCHEZ et al., 2003).

Objetivos gerais

OBJETIVOS GERAIS

Este trabalho teve como objetivos gerais:

1. Aumentar o conhecimento sobre o veneno de *C. d. cascavella* e sua crotoxina, suas ações neurotóxica e miotóxica.
2. Produzir anticorpos contra a crotoxina, a PLA_2 (subunidade tóxica) e crotapotina (subunidade não tóxica).
3. Avaliar a capacidade destes anticorpos em neutralizar a neurotoxicidade, a miotoxicidade e a atividade enzimática da crotoxina e da PLA_2.
4. Avaliar a reatividade cruzada destes anticorpos frente aos venenos de *C. d. terrificus* e *B. jararacussu*.

Capítulo 1

"Neurotoxic and myotoxic actions of crotoxin-like and *Crotalus durissus cascavella* whole venom in the chick biventer cervicis preparation"

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Neurotoxic and myotoxic actions of crotoxin-like and *Crotalus durissus cascavella* whole venom in the chick biventer cervicis preparation

Daniela G. Beghini[a], Léa Rodrigues-Simioni[c], M.H. Toyama[a], J.C. Novello[a], Maria Alice da Cruz-Höfling[b,*], S. Marangoni[a]

[a]*Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CEP 13083-970, P.O. Box 6109, Campinas, SP, Brazil*

[b]*Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CEP 13083-970, P.O. Box 6109, Campinas, SP, Brazil*

[c]*Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), CEP 13083-970, P.O. Box 6109, Campinas, SP, Brazil*

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Abstract

Crotoxin from *Crotalus durissus cascavella* venom was purified by a combination of molecular exclusion chromatography (Superdex 75 column) and HPLC molecular exclusion (Protein Pack 300SW column). Neurotoxic and myotoxic effects from *C. durissus cascavella* whole venom and its main fraction, the crotoxin-like, were studied in the chick biventer cervicis (CBC) nerve–muscle preparation. Both venom and its crotoxin showed significant ($p < 0.05$) blockade of neuromuscular transmission at concentrations as low as 0.2–1, 5 and 25 μg/ml, but no significant effect has been shown with a concentration of 0.04 μg/ml ($n = 5$ each). The time required to produce 50% neuromuscular blockade with the venom and its crotoxin was 53.6 ± 8.2 and 65.9 ± 4.9 min (0.2 μg/ml), 29.7 ± 1.9 and 34.3 ± 1.9 min (1 μg/ml), 24.8 ± 1.6 and 21.1 ± 1.5 min (5 μg/ml), 20.9 ± 3.7 and 20.1 ± 1.4 min (25 μg/ml), respectively. The addition to the incubation bath of acetylcholine (55 and 110 μM) or KCl (20.1 mM), either before or after the venom or the crotoxin induced contracture in the presence of a total blockade, in all the concentrations used. Morphological analysis showed that the damage caused by *C. durissus cascavella* venom is stronger than that caused by crotoxin. The myonecrotic picture was more marked at higher venom and crotoxin doses (1, 5 or 25 μg/ml). Only at 25 μg/ml concentrations of the venom and crotoxin, marked muscle fiber changes were detected. We concluded that the crotoxin-like and the whole venom from *C. durissus cascavella* possess a preponderant and quite potent neurotoxic action in this preparation, and a myotoxic action which is observed only at higher doses.

Keywords: Neuromuscular blockade; Acetylcholine; KCl; Neurotoxicity; Myotoxicity

1. Introduction

Crotoxin is the main neurotoxic component of the South American *Crotalus durissus terrificus* rattlesnake venom. Besides the crotoxin (Slotta and Fraenkel-Conrat, 1938), proteins such as gyroxin (Barrio, 1961), crotamine (Gonçalves and Vieira, 1950) and convulxin (Prado-Franceschi and Vital Brazil, 1981) are also present in the venom. Crotoxin accounts for approximately 50% of the venom proteins and it is, clinically, the most important. It has neurotoxic (Vital Brazil, 1966), myotoxic (Azevedo-Marques et al., 1982; Cupo et al., 1988), hemolytic

* Corresponding author. Tel.: +55-19-3788-6247; fax: +55-19-3289-3124.

E-mail address: hofling@unicamp.br (M.A. da Cruz-Höfling).

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(Rosenfeld, 1971) and platelet-aggregating (Landucci et al., 1994) activities.

Crotoxin is a presynaptic neurotoxin (Vital Brazil and Excell, 1971; Hawgood and Smith, 1977), which contains basic phospholipase A_2 and an acidic protein, crotapotin. Crotapotin is non-toxic and the phospholipases A_2 are less toxic than the crotoxin complex (Rübsamen et al., 1971). The crotoxin blocks neuromuscular transmission (Hawgood and Smith, 1977) and at high concentrations may cause myonecrosis (Gopalakrishnakone and Hawgood, 1984). Crotoxin from *C. durissus terrificus* venom has been studied extensively, although crotoxin proteins from other *C. durissus* venoms, such as *Crotalus durissus cascavella*, have been poorly studied regarding their neurotoxic and myotoxic effects. *C. durissus cascavella* (maracambóia) is a medically important rattlesnake species in northeastern Brazil and northern Amazon state (Martins et al., 1998). The phospholipase A_2 and crotapotin's subunits of crotoxin from the *C. durissus cascavella* venom have recently been characterized by Beghini et al. (2000).

In this work, we have isolated crotoxin from *C. durissus cascavella* venom with a two-step procedure starting with Superdex 75, followed by a second step of exclusion molecular chromatography on HPLC (Protein Pack 300SW) to study the neurotoxic and myotoxic effects of the venom, and its crotoxin in the CBC preparation. Neurotoxicity was assessed by the ability of venom and crotoxin-like to cause neuromuscular blockade, whereas myotoxicity was assessed by the ability to cause muscle fiber damage, as seen by light microscopy. Some discussion will also be addressed to the comparison of these effects to the ones induced by *C. durissus terrificus* venom and its crotoxin.

2. Materials and methods

2.1. Venom and toxin

Desiccated *C. durissus cascavella* venom was purchased from a private serpentarium (Batatais, SP, Brazil). The water, buffers and solvents used in the purification protocols were filtered through 0.22 μm filters (Millipore) and degassed by sonication.

2.2. Molecular exclusion chromatography

The whole venom from *C. durissus cascavella* (35 mg) was dissolved in 0.2 ml of ammonium bicarbonate buffer (1.0 M, pH 8.0). The sample was then applied onto a Superdex 75 column (1×60 cm^2) using LC 650E (Waters). The column was previously equilibrated with ammonium bicarbonate buffer (0.2 M, pH 8.0) and eluted with the same buffer to a constant flow of 0.3 ml/min. Elution profile was monitored at 280 nm and the fractions corresponding to crotoxin were sampled, lyophilized and stored at −20 °C.

2.3. HPLC molecular exclusion chromatography

Crotoxin recovered from Superdex 75 column was further purified on Protein pack 300SW column (0.78×30 cm^2) and eluted with 0.1 M ammonium bicarbonate, pH 7.9, at a flow rate of 0.1 ml/min. The elution profile was monitored at 280 nm using a 490 programmable multi-wavelength UV/visible detector. The chromatographic runs were recorded with a 746 data modular channel recorder (Waters Corp.). The fraction corresponding to crotoxin was lyophilized and stored at −20 °C.

2.4. Animals

Male chicks (4–8 days old, HY-LINE W36 lineage) were supplied by Granja Ito S/A (Campinas, SP, Brazil). Animals had free access to food and water.

2.5. Biventer cervicis muscle preparation

Male chicks were anesthetized with chloral hydrate (3 mg/kg) and sacrificed by exsanguination. The biventer cervicis muscles were removed (Ginsborg and Warriner, 1960) and mounted under a tension of 1 g, in a 5 ml organ bath containing warmed (37 °C), aerated (95%O_2–5%CO_2) Krebs solution (pH 7.5) of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25.0 and glucose 11.65. Contracture to exogenously applied acetylcholine (ACh 55 and 110 μM for 60 s) and KCl (20.1 mM for 130 s) were obtained in the absence of field stimulation, prior to the addition of the single dose (0.04, 0.2, 1, 5 or 25 μg/ml) of the venom or crotoxin and at the end of the experiment, as a further test for the presence of myotoxic and neurotoxic activities (Harvey et al., 1994). A bipolar platinum ring electrode was placed around the tendon within which runs the nerve trunk supplying the muscle. Indirect stimulation was performed with a Grass S48 stimulator (0.1 Hz, 0.2 ms, 3–4 V). Muscle contractions and contractures were recorded isometrically via a force–displacement transducer (BG-10GM Kulite) coupled to a Gould RS3400 record. In some preparations, neostigmine (1–4 μg/ml) was added after complete neuromuscular blockade induced by venom or crotoxin-like fraction.

2.6. Histological examination

After the total neuromuscular blockade or after 120 min incomplete neuromuscular blockade by *C. durissus cascavella* venom or crotoxin (control preparations were incubated in Krebs solution for 120 min, $n = 3$), the CBC muscle preparations were fixed in Bouin solution for 24–48 h followed by dehydration and embedding in historesin. Cross-sections with 5 μm thick were stained

with hematoxylin–eosin (HE) prior to examination by light microscopy.

2.7. Statistical analysis

Each experimental protocol was repeated at least five times, and the results reported as the mean ± SEM. ANOVA followed by the Bonferroni test was used for statistical comparison of the data. A value of $p < 0.05$ was considered to indicate significance.

3. Results

3.1. Molecular exclusion chromatography

The elution profile of *C. durissus cascavella* venom following molecular exclusion chromatography on Superdex 75 column showed two main fractions (I and III) corresponding to convulxin and crotoxin, respectively (Fig. 1A). The pharmacological activity of these fractions was evaluated, the fraction III being responsible for paralysis of the mouse diaphragm (not shown), as usual for crotoxin from *C. durissus terrificus* in rat (Vital Brazil, 1966) and mouse (Oshima-Franco et al., 1999) diaphragm preparations. Fraction III was re-chromatographed on an HPLC Protein Pack 300SW column resulting in one major peak which was called crotoxin-like (Fig. 1B). The biological activity of this peak was assayed in CBC muscle preparation.

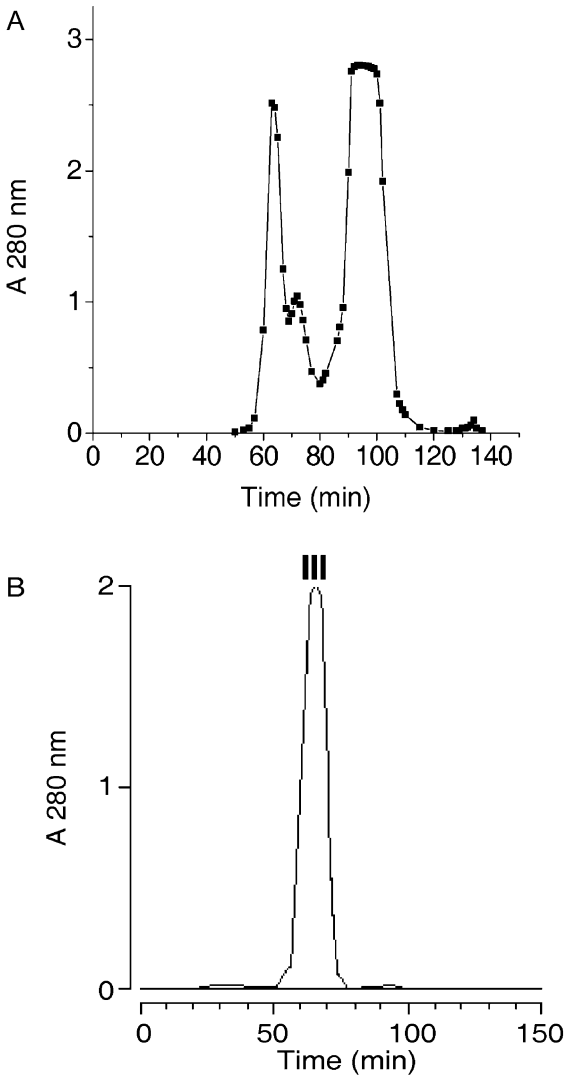

Fig. 1. (A) Elution profile of *C. durissus cascavella* venom by molecular exclusion chromatography on Superdex G75 column. (B) Elution profile of fraction III of crotoxin-like from *C. durissus cascavella* by HPLC molecular exclusion chromatography (Protein Pack 300SW).

3.2. Neuromuscular blockade

The venom and crotoxin (0.2, 1 and 5 μg/ml) caused a dose-dependent blockade of the neuromuscular transmission (Fig. 2A and B). Very small blocking effect was observed using 0.04 μg/ml. Venom or crotoxin concentrations of 5 μg/ml represented the maximum dose for a neuromuscular blockade. The time required for a 50% reduction of the twitch tension through an indirect stimulation by the venom and its crotoxin-like of *C. durissus cascavella* was 53.6 ± 8.2 and 65.9 ± 4.9 min (0.2 μg/ml), 29.7 ± 1.9 and 34.3 ± 1.9 min (1 μg/ml), 24.8 ± 1.6 and 21.1 ± 1.5 min (5 μg/ml), 20.9 ± 3.7 and 20.1 ± 1.4 min (25 μg/ml), respectively ($n = 5$/dose). Doses as high as 0.2, 1, 5 and 25 μg/ml of the whole venom and crotoxin-like elicited a total and irreversible twitch tension blockade (Fig. 2A and B), which was maintained even after washing it over for three times, or after the addition of an anticholinesterasic drug (neostigmine 1–4 μg/ml). In the control preparations, the twitch tension records remained stable (93.9% all along the 120 min of incubation in Krebs solution, $n = 5$).

The whole venom or crotoxin, independent of the concentration studied, did not significantly alter the ACh and KCl-induced contractures when compared to the control values (Fig. 3A and B). In the control preparations, the contracture to ACh and KCl kept stable, after a 120 min indirect stimulation when compared to the control values (data not shown).

3.3. Morphological changes

The normal histological appearance of CBC muscle incubated with Krebs solution consisted of peripheral nuclei and well-organized fibers with regular visible transversal striations (Fig. 4). When incubated with *C. durissus cascavella* venom or crotoxin-like fraction either at 0.04

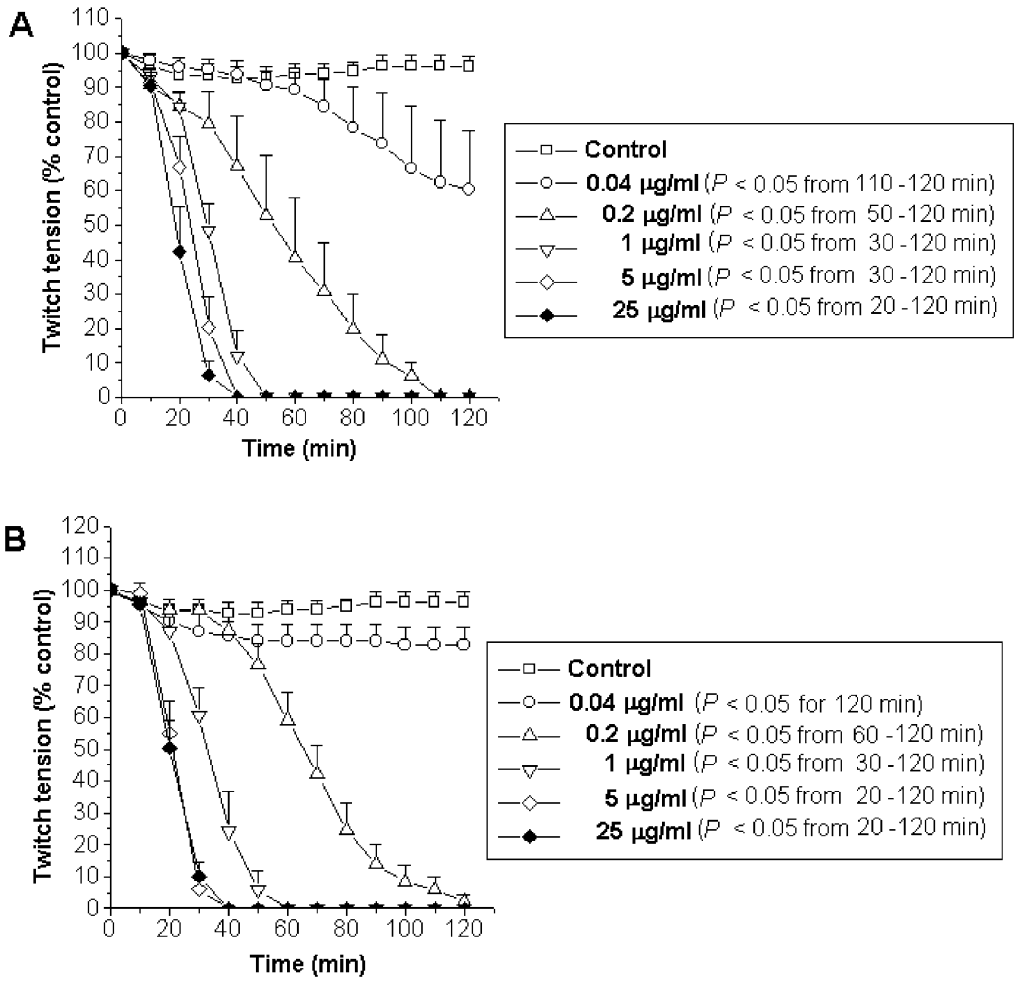

Fig. 2. Neuromuscular blockade induced by the total venom (A) and crotoxin-like (B) from *C. durissus cascavella* on the CBC preparation. Each point represents the average from five experiments ± EPM. $p < 0.05$ compared to control.

or 0.2 μg/ml, the CBC preparation muscle showed fibers with normal morphology. When incubated with *C. durissus cascavella* venom or crotoxin-like fraction at 1, 5 μg/ml, areas with intact fibers might present some darker in color, and hypercontracted fibers with cross-striations were ill-defined in the middle. Only when 25 μg/ml of both the venom (Fig. 5A) and fraction (Fig. 5B) was used it was possible to infer indirectly by light microscopy that there were signs of ruptured sarcolemma, as inferred by the myonecrotic picture of the tissue. The myonecrosis was manifested in a large number of muscle fibers and included discontinuous fiber outline, myofibrils fragmentation and densely clumped myofibrils in different stages of aggregation. Although no quantitative evaluation has been made, the qualitative results obtained clearly indicate that the myotoxic effect seen through light microscopy in the CBC preparation was dependent on the venom dose. The myotoxic activity caused by *C. durissus cascavella* venom (25 μg/ml) is stronger than that caused by crotoxin (25 μg/ml) in the CBC preparation.

4. Discussion

The protocol generally employed for the purification of rattlesnake neurotoxins starts with Sephadex G75, followed by a second step of gel filtration on Sephadex G25 or either anion or cation exchange chromatography (Faure and Bon, 1988). Crotoxin from *C. durissus cascavella* venom had been purified by HPLC molecular exclusion and its subunits were purified by reverse-phase HPLC (Beghini et al., 2000). In this work, we purified the crotoxin from *C. durissus cascavella* whole venom using two chromatographic steps. In the first step (Superdex 75 column), toxins other than

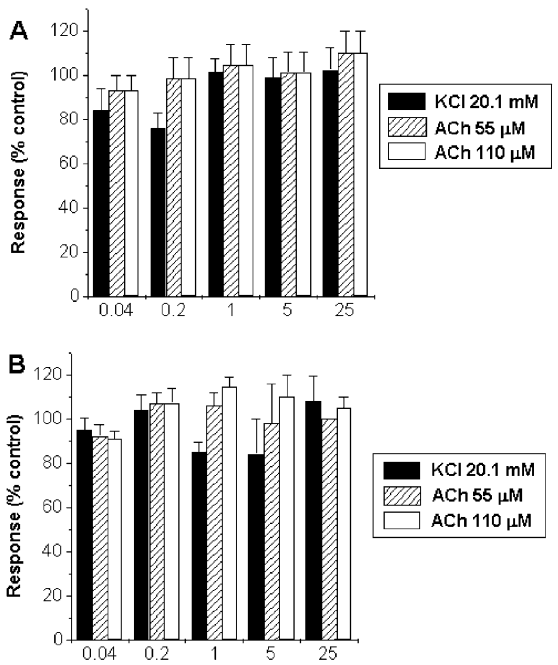

Fig. 3. Effect of the total venom (A) and crotoxin-like (B) from *C. durissus cascavella* on the response of the CBC preparation to the acetylcholine (ACh) and potassium (KCl). The preparation was exposed to the venom and crotoxin-like, in the suitable concentrations, by the numbers (μg/ml) placed under each histogram. Each point represents the average of five experiments ± EPM.

crotoxin-like were eliminated from the whole venom; in the second (molecular exclusion chromatography in HPLC-Protein pack 300SW), a single peak of crotoxin purified was obtained. This purification protocol was simple, rapid, efficient and resulted in a better final yield for crotoxin.

The crotoxin induced its lethal action through the blockade of the neuromuscular transmission (Vital Brazil, 1966; Chang and Lee, 1977). Besides, muscle damage is one

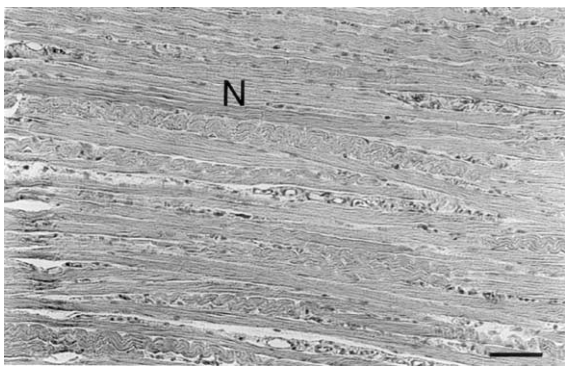

Fig. 4. Normal (control) CBC muscle (longitudinal section) shows the normal histological appearance. Note the presence of regular transversal striations (*N*) after 120 min incubation with Krebs solution. Bar = 60 μm.

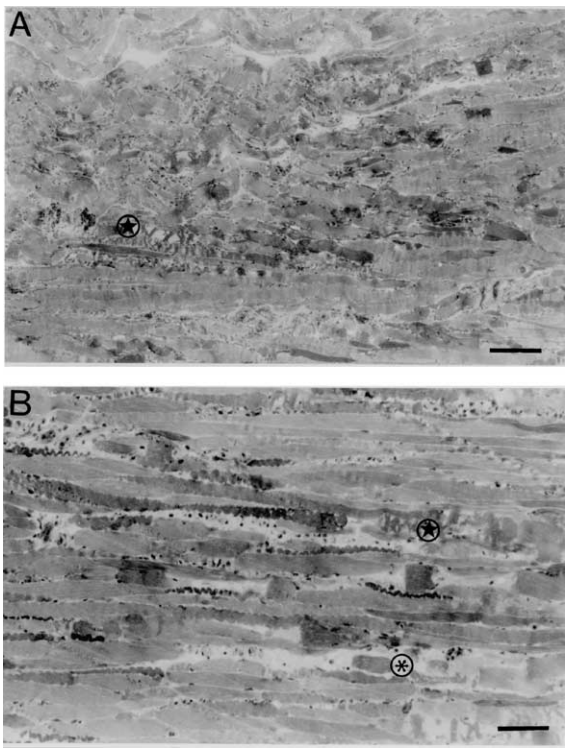

Fig. 5. Panels A and B show light micrographs of CBC muscle preparation (longitudinal section), incubated with *C. durissus cascavella* venom and its homologous crotoxin (25 μg/ml), respectively. The myonecrotic picture was stronger for the venom than for the fraction, but both showed fragmented myofibrils (*) and by condensed multi-shaped myofibril agglutinations (★). Bars = 60 μm in all panels.

of the most common symptoms produced by venoms from species in the Viperidae family. Venom components having a specific direct action on skeletal muscle cells are called myotoxins (Mebs and Ownby, 1990). Our findings showed that the *C. durissus cascavella* venom reported here and its crotoxin-like component, presented a quite potent neurotoxic activity even at lower doses. Indeed, the doses of 0.2, 1 and 5 μg/ml of the whole venom and of crotoxin from *C. durissus cascavella* induced a total and irreversible twitch tension blockade, indicating a potent neurotoxic activity in the CBC preparation. Regarding the myotoxic activity of the venom and its crotoxin-like, it was seen that an extensive muscular damage occurred only when 25 μg/ml was used. At 0.04, 0.2 and 1 μg/ml concentrations of the venom and crotoxin, no muscle fiber changes were detected. At 5 μg/ml the myotoxic action of both venom and its crotoxin was mild. Such findings indicate that the myotoxicity is not the preponderant effect and most probably has very little effect, if any, on the neuromuscular blockade.

A presynaptically active neurotoxin would abolish nerve-evoked twitches, without affecting response to cholinoreceptor agonists. Postsynaptically active neurotoxins would

block response to cholinoreceptors agonists. The fact that crotoxin and the whole venom from *C. durissus cascavella* did not significantly affect ($p < 0.05$) the responses to ACh and KCl added in the preparation agrees with what is known about the interference of the *C. durissus terrificus* venom on ACh release (Hawgood and Smith, 1977; Hawgood and Santana de Sá, 1979), i.e. through a presynaptic action. In agreement, the electrophysiological analyses on the effect of the crotoxin from *C. durissus terrificus* on various preparations, including frog sartorius, mouse, rat and cat diaphragms and chicken biventer cervicis, have indicated that crotoxin interferes with the transmitter release (Marlas and Bon, 1982). Our results showed a presynaptic action of *C. durissus cascavella* venom and its crotoxin-like, agreeing with the above authors' results for the *C. durissus terrificus* venom and its crotoxin. However, the response of rat-denervated diaphragm to acetylcholine was reduced after exposure to the crotoxin, which suggested a postsynaptic action (Vital Brazil, 1966). Therefore, whether *C. durissus cascavella* venom and crotoxin-like also possess a similar postsynaptic action is yet to be elucidated.

It is expected that the presence of the myotoxic components in the snake venoms would reduce the contracture response of the skeletal muscle to the addition of high concentration of potassium (Harvey et al., 1994). As seen in the results, after the neuromuscular blockade has been completed, the muscle continued to respond to potassium addition, even with as high as 25 μg/ml, a concentration of the venom or crotoxin-like in which an extensive myonecrosis has been observed. This finding shows that there is not a direct parallelism between morphological damage and functional change at this dose. The myotoxicity caused by *C. durissus cascavella* venom was dose-dependent and more easily detected than crotoxin-like. With equal doses, the total venom presented larger myotoxic potency than its crotoxin component. Two types of myotoxic agents are found in Crotalid venoms: polypeptides like crotamine (Vital Brazil et al., 1979) and myotoxin *a* (Ownby et al., 1976) (both of which are free of enzymatic activity), and phospholipases A_2 (Gopalakrishnakone et al., 1984). However, in the present study the crude venom from *C. durissus cascavella*, without the crotamine presence (Beghini et al., 2000; Santoro et al., 1999), seemed to be more efficient in inducing myotoxicity than its crotoxin component. Components other than the phospholipase A_2 (crotoxin) of the *C. durissus cascavella* venom, with potential to produce myotoxicity, should be present in the venom and remain to be elucidated. It has been reported that *C. durissus cascavella* venom presents a significant higher phospholipase A_2 activity than *C. durissus collilineatus*, which could account for this toxicity (Santoro et al., 1999).

To conclude, the crotoxin-like and the whole venom of *C. durissus cascavella* possess a quite potent primarily neurotoxic action visible in concentrations as low as 0.2 μg/ml and a secondary myotoxic action detected only at higher doses in the CBC preparation.

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Capítulo 2

"Anti-sera raised in rabbits against crotoxin and phospholipase A_2 from *Crotalus durissus cascavella* venom neutralize the neurotoxicity of the venom and crotoxin"

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Anti-sera raised in rabbits against crotoxin and phospholipase A_2 from *Crotalus durissus cascavella* venom neutralize the neurotoxicity of the venom and crotoxin

Daniela G. Beghini[a], Saraguaci Hernandez-Oliveira[b], Léa Rodrigues-Simioni[b], José Camilo Novello[a], Stephen Hyslop[b], Sérgio Marangoni[a,*]

[a]*Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970 Campinas, SP, Brazil*

[b]*Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), CEP 13083-970 Campinas, SP, Brazil*

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Abstract

Crotoxin, the principal neurotoxin in venom of the South American rattlesnakes *Crotalus durissus terrificus* and *Crotalus durissus cascavella*, contains a basic phospholipase A_2 (PLA_2) and an acidic protein, crotapotin. In this work, we examined the ability of rabbit anti-sera against crotoxin and its PLA_2 subunit to neutralize the neurotoxicity of venom and crotoxin from *C. d. cascavella* in mouse phrenic nerve–diaphragm and chick biventer cervicis preparations. Immunoblotting showed that the anti-sera recognized *C. d. cascavella* crotoxin and PLA_2. This was confirmed by ELISA, with both anti-sera having end-point dilutions of 3×10^{-6}. Anti-crotoxin serum neutralized the neuromuscular blockade in phrenic nerve–diaphragm muscle preparations at venom or crotoxin:anti-serum ratios of 1:2 and 1:3, respectively. Anti-PLA_2 serum also neutralized this neuromuscular activity at a venom or crotoxin:anti-serum ratio of 1:1. In biventer cervicis preparations, the corresponding ratio for anti-crotoxin serum was 1:3 for venom and crotoxin, and 1:1 and 1:2 for anti-PLA_2 serum. The neutralizing capacity of the sera in mouse preparations was comparable to that of commercial anti-serum raised against *C. d. terrificus* venom. These results show that anti-sera against crotoxin and PLA_2 from *C. d. cascavella* venom neutralized the neuromuscular blockade induced by venom and crotoxin in both nerve–muscle preparations, with the anti-serum against crotoxin being slightly less potent than that against crotoxin.

Keywords: Anti-serum; Crotapotin; Crotoxin; Neuromuscular blockade; Neutralization; Phospholipase A_2

1. Introduction

Crotoxin, the major toxin of South American rattlesnake (*Crotalus durissus terrificus*) venom (Slotta and Fraenkel-Conrat, 1938), is a potent phospholipase A_2 neurotoxin that produces neuromuscular blockade (Vital Brazil, 1966). Several additional biological activities, including myotoxicity (Azevedo-Marques et al., 1982), hemolysis (Rosenfeld, 1971) and platelet aggregating activity (Landucci et al., 1994), have also been attributed to crotoxin.

Crotoxin consists of a basic, weakly toxic phospholipase A_2 (PLA_2) subunit and an acidic, non-toxic subunit (crotapotin) devoid of enzymatic activity (Hendon and Fraenkel-Conrat, 1971; Breithaupt, 1976). Crotapotin acts as a chaperon in the complex by preventing non-specific binding of the PLA_2, thereby potentiating its toxicity (Bon et al., 1979). Thus, the stability of the interaction between

* Corresponding author. Tel.: +55-19-3788-6134; fax: +55-19-3788-6129.

E-mail address: marango@unicamp.br (S. Marangoni).

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the PLA_2 and crotapotin plays a major role in the toxicity of crotoxin (Faure et al., 1993). Binding experiments have shown that the two subunits of crotoxin separate upon interaction with biological membranes; with the PLA_2 binding to membranes while crotapotin is released into solution. In the absence of crotapotin, the PLA_2 adsorbs to membranes in a non-saturable manner, whereas in the presence of crotapotin the PLA_2 binds to a limited number of high-affinity binding sites (Jeng et al., 1978; Bon et al., 1979).

Santoro et al. (1999) reported that the composition and biological activities of *Crotalus durissus cascavella*, *Crotalus durissus collilineatus* and *C. d. terrificus* venoms were very similar, except for minor differences in paw edema-inducing activity, electrophoretic profile, phospholipase A_2 activity, crotamine-like activity and inorganic element contents of *C. d. cascavella* venom. Crotoxin is the principal neurotoxin in the venoms of these subspecies. Whereas the immunological properties of crotoxin and its subunits from *C. d. terrificus* venom have been extensively studied (Dos Santos et al., 1988; Faure et al., 1993), little is known about these properties of crotoxin from the venoms of other *C. durissus* subspecies, including *C. d. cascavella*. In the present study, we examined the ability of anti-sera raised in rabbits against crotoxin (anti-crotoxin) and PLA_2 (anti-PLA_2) from *C. d. cascavella* venom to neutralize the neurotoxicity of this venom and its crotoxin. Neurotoxicity was assessed by the ability of venom and crotoxin to cause neuromuscular blockade in electrically stimulated mouse phrenic nerve–diaphragm and chick biventer cervicis preparations. Neutralization was tested by preincubating the anti-sera with venom or crotoxin and then assaying the residual biological activity.

2. Materials and methods

2.1. Venom and toxin

Desiccated *C. d. cascavella* venom was purchased from a private serpentarium (Batatais, SP, Brazil). Crotoxin and PLA_2 were purified from *C. d. cascavella* venom as described by Beghini et al. (2000).

2.2. Animals

Male Swiss white mice (26–32 g) were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). The mice were housed at 25 °C on a 12 h light/dark cycle and had free access to food and water. Male chicks (4–8 days old, HY-LINE W36 lineage) were supplied by Granja Ito S/A (Campinas, SP, Brazil) and had free access to food and water. Male New Zealand white rabbits (2–3 kg) were purchased from an established breeder (Granja de Coelhos Grota Azul, Paulínia, SP) and housed individually with free access to food and water. The experiments described here were done within the guidelines established by the Brazilian College for Animal Experimentation (COBEA).

2.3. Anti-venom

The commercial crotalic anti-venom used was a semi-purified immunoglobulin-rich solution produced by hyperimmunization of horses with *C. d. terrificus* venom (Instituto Vital Brazil, Rio de Janeiro, RJ, Brazil). This anti-venom is distributed for use after peptic digestion and partial purification by ammonium sulfate precipitation, and contains 0.35% phenol as preservative.

2.4. Anti-sera

Rabbits were immunized by successive i.m. and i.d. inoculations with 500 μg of crotoxin or 750 μg of PLA_2 from crotoxin per rabbit. The first injections were i.m. and included Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA) in a 1:1 ratio. The subsequent boosters were given i.d. with the toxin dissolved in 0.9% (w/v) NaCl. The antibody levels in the sera were monitored by gel immunodiffusion (Ouchterlony, 1949) and ELISA (Chavez-Olórtegui et al., 1993). Blood samples were collected from a marginal ear vein and stored at 4 °C overnight. The sera were subsequently separated by centrifugation and aliquots were stored at −70 °C.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (96 wells) were coated overnight at 4 °C with 100 μl of venom, crotoxin or PLA_2 (5 μg/well) in sodium bicarbonate buffer. The plates were washed three times with saline solution containing 0.5% Tween 20 and unbound sites were blocked for 1 h at room temperature with 2% bovine casein in phosphate-buffered saline (PBS). The plates were again washed (three times) with saline solution containing 0.5% Tween 20 and used immediately for ELISA. To measure the serum titers, 100 μl of serial dilutions of serum in PBS containing 0.25% bovine casein and 0.05% Tween 20 were added to the plates and incubated for 1 h at room temperature. The plates were washed again and incubated for 1 h with 100 μl of a goat anti-rabbit immunoglobulin G (whole molecule)–peroxidase conjugate (Sigma, St Louis, MO, USA; 1:1000 in PBS containing 0.25% bovine casein and 0.05% Tween 20), followed by further washing. The substrate solution for the peroxidase assay (citrate buffer, pH 5.0, containing 0.2 mg of *o*-phenylenediamine/ml and 0.5 μl of 30% H_2O_2/ml) was added and the enzymatic reaction allowed to proceed for 15 min in the dark at room temperature. The reaction was stopped with 50 μl of 5% H_2SO_4 and the absorbance was read at 492 nm with a SpectraMax 340 multi-well plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.6. Immunoblotting

Crotoxin and PLA_2 (10 or 15 μg) were run on 12.5% polyacrylamide minigels in SDS-PAGE (Hoefer SE 260 Mighty Small™ system) at 100 V for 2 h and the proteins then transferred electrophoretically to nitrocellulose membranes (0.45 μm) in a TE-22 minitransfer tank (Hoefer-Pharmacia Biotech, Inc., San Francisco, CA, USA) at 400 mA for 1 h. Subsequently, the membranes were blocked at room temperature for 2 h in a solution of 5% non-fat milk/0.05% Tween 20. After washing six times in Tris-buffered saline (TBS; 0.01 M Tris–HCl, 0.17 M NaCl, pH 7.6), the membranes were incubated overnight with rabbit anti-serum (anti-crotoxin or anti-PLA_2) (diluted 1:5000 in TBS) or commercial anti-venom (diluted 1:1500 in TBS). After washing again with TBS, bound antibodies were detected with a goat anti-rabbit immunoglobulin G (whole molecule)–peroxidase conjugate (Sigma; 1:1000 in TBS) for rabbit anti-serum or with a rabbit anti-horse immunoglobulin G (whole molecule)–peroxidase conjugate (Sigma; 1:1500 in TBS) for commercial anti-venom for 2 h at room temperature with shaking. At the end of this incubation, the blots were washed, developed with 4-chloro-1-naphthol (Sigma; 0.03% in 0.05 M Tris–HCl, pH 7.6, containing 0.03% H_2O_2) and documented.

2.7. Mouse phrenic nerve–diaphragm muscle preparation

The phrenic nerve and diaphragm (Bülbring, 1946) were obtained from mice anesthetized with chloral hydrate (300 mg/kg, i.p.) and sacrificed by exsaguination. The diaphragm was removed and mounted under a tension of 5 g in a 5 ml organ bath containing Tyrode solution (pH 7.4, 37 °C) of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 11.1, aerated with 95% O_2 and 5% CO_2. Supramaximal stimuli (4 × threshold, 0.1 Hz, 0.2 ms) delivered from a Grass S48 stimulator (Astro-Med, Inc., W. Warwick, RI) were applied to the nerve through bipolar electrodes. Isometric muscle tension was recorded by a force displacement transducer (Load Cell BG-10 GM, Kulite Semiconductor Products, Inc., NJ) coupled to a physiograph (Gould, Model RS 3400) via a Gould universal amplifier (both from Gould, Inc., Recording Systems Division, Cleveland, OH). The preparations were allowed to stabilize for at least 20 min before the addition of *C. d. cascavella* venom or crotoxin (10 μg/ml) or of a mixture of venom or crotoxin that had been preincubated with the desired volume of rabbit anti-serum or commercial anti-venom for 30 min at 37 °C. In the latter cases, the final venom or crotoxin concentrations were the same as in the experiments without anti-serum. Control experiments were also done using Tyrode solution with anti-serum or anti-venom.

2.8. Chick biventer cervicis muscle preparation

Male chicks were anesthetized with chloral hydrate (3 mg/kg, i.p.) and sacrificed by exsanguination. The biventer cervicis muscles were removed (Ginsborg and Warriner, 1960) and mounted under a tension of 0.5 g in a 5 ml organ bath containing warmed (37 °C), aerated (95%O_2–5%CO_2) Krebs solution (pH 7.5) of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25.0 and glucose 11.65. A bipolar platinum ring electrode was placed around the tendon within which runs the nerve trunk supplying the muscle. Indirect stimulation was done with a Grass S48 stimulator (0.1 Hz, 0.2 ms, 3–4 V). Muscle contractions and contractures were recorded isometrically via a force–displacement transducer (BG-10GM Kulite) coupled to a Gould RS3400 recorder. The preparations were allowed to stabilize for at least 20 min before the addition of *C. d. cascavella* venom or crotoxin (5 μg/ml) or of a mixture of venom or crotoxin that had been preincubated with the desired volume of rabbit anti-serum for 30 min at 37 °C. In the latter cases, the final venom or crotoxin concentrations were the same as in experiments without anti-serum. Control experiments were done using Krebs solution with anti-serum.

2.9. Statistical analysis

Each experimental protocol was repeated 3–5 times, and the results were expressed as the mean ± SEM. ANOVA followed by the Bonferroni test was used for statistical comparison of the data. A value of $P < 0.05$ indicated significance.

3. Results

3.1. Antibody production

Antibody production during the immunization process was monitored by double immunodiffusion until strong immunoprecipitin bands were consistently obtained (results not shown), at which point the rabbits were bled and the antibody titers of the sera determined by ELISA. Fig. 1 shows the reactivity of crotoxin, PLA_2 and *C. d. cascavella* venom with serial dilutions of the anti-crotoxin and anti-PLA_2 sera, and with commercial anti-venom. In all cases, the reactivities were greatest with the corresponding immunogen (crotoxin, PLA_2 and venom for anti-crotoxin, anti-PLA_2 and commercial sera, respectively). The endpoint dilutions of the anti-crotoxin and anti-PLA_2 sera were similar (3×10^6), and greater than for commercial anti-venom (10^5–10^6).

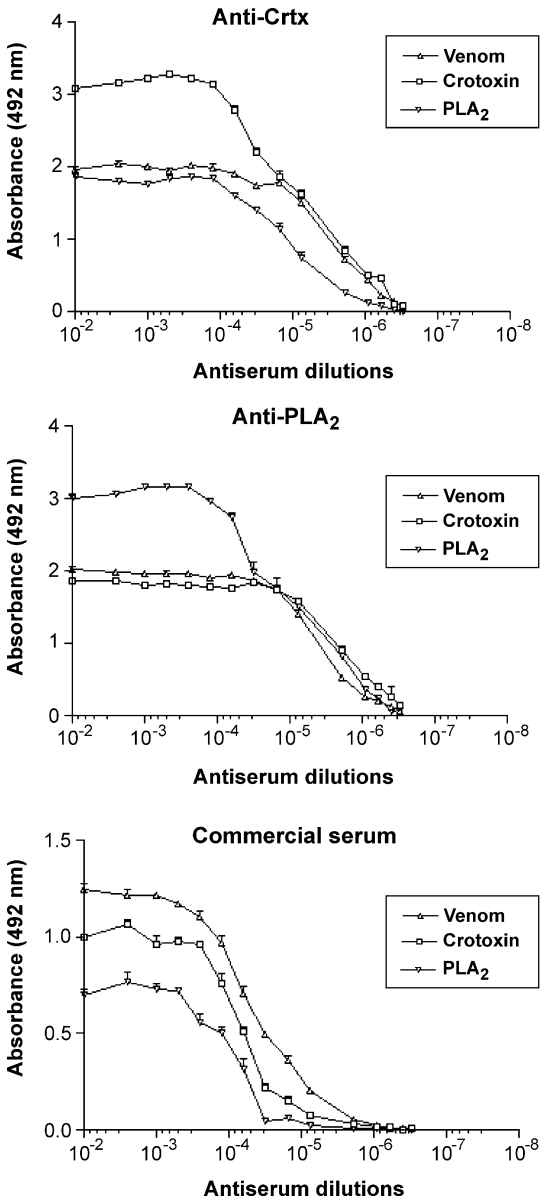

Fig. 1. ELISA reactivity of anti-crotoxin, anti-PLA_2 and commercial anti-sera with venom, crotoxin and PLA_2 from *C. d. cascavella*. The plates were coated with antigen (5 μg/well) then incubated with anti-serum at the dilutions indicated and finally with an appropriate IgG–peroxidase conjugate and substrate (OPD). The resulting absorbances were read at 492 nm and each point is the mean ± SEM of four determinations.

3.2. Immunoblotting

Fig. 2 shows immunoblots of *C. d. cascavella* crotoxin and PLA_2 detected with anti-PLA_2 and anti-crotoxin sera (diluted 1:5000), and with commercial anti-venom (diluted 1:1500). All of the anti-sera recognized crotoxin and PLA_2 from *C. d. cascavella* venom.

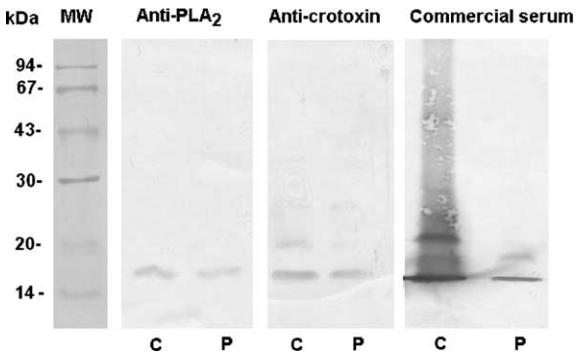

Fig. 2. Immunoblot showing the reactivity of anti-crotoxin, anti-PLA_2 and commercial anti-sera with crotoxin (C) and PLA_2 (P) from *C. d. cascavella*. The molecular weight (MW) standards were used: phosphorilase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). The electrophoresis and transfer of proteins were done as described in Section 2.6. The amount of protein applied to each lane was 10 μg for blots with rabbit anti-sera and 15 μg for commercial anti-serum.

3.3. Neuromuscular activity and its neutralization

Neuromuscular activity was assayed using single concentrations of *C. d. cascavella* venom or crotoxin in the mouse phrenic nerve–diaphragm (10 μg/ml each) and chick biventer cervicis (5 μg/ml each) preparations. These concentrations were chosen based on preliminary experiments in which they produced neuromuscular blockade within a reasonable timescale. The times required for 50% neuromuscular blockade in the mouse preparations were 35.9 ± 1.1 min ($n = 5$) and 41.2 ± 3.9 min ($n = 5$) for the venom and crotoxin, respectively (each at 10 μg/ml). In the chick preparations, the times for 50% neuromuscular blockade at 5 μg/ml were 24.8 ± 1.6 min ($n = 5$) and 21.1 ± 1.5 min ($n = 5$) for the venom and crotoxin, respectively.

3.3.1. Phrenic nerve–diaphragm preparation

Fig. 3A shows that although the anti-sera alone caused a slight reduction (maximum of 15% with commercial anti-venom) in muscle contractility, this effect was not significantly different from the basal neuromuscular response. Fig. 3B shows the neutralization of venom-induced neuromuscular blockade by rabbit anti-sera to crotoxin and PLA_2 and by commercial anti-venom. Anti-serum to crotoxin protected against the neuromuscular blockade by venom at a venom:anti-serum ratio ≥1:2 ($P < 0.05$ from 20 min post-venom onwards, $t_{20-120\ min}$). Likewise, anti-serum to PLA_2 protected against the venom-induced neuromuscular blockade at a venom:anti-serum ratio ≥1:1 ($P < 0.05$, for $t_{10-120\ min}$). Commercial crotalic anti-venom protected against the neuromuscular blockade at a venom:anti-venom ratio ≥1:1 ($P < 0.05$, for $t_{30-120\ min}$). None of the anti-sera offered significant protection at ratios

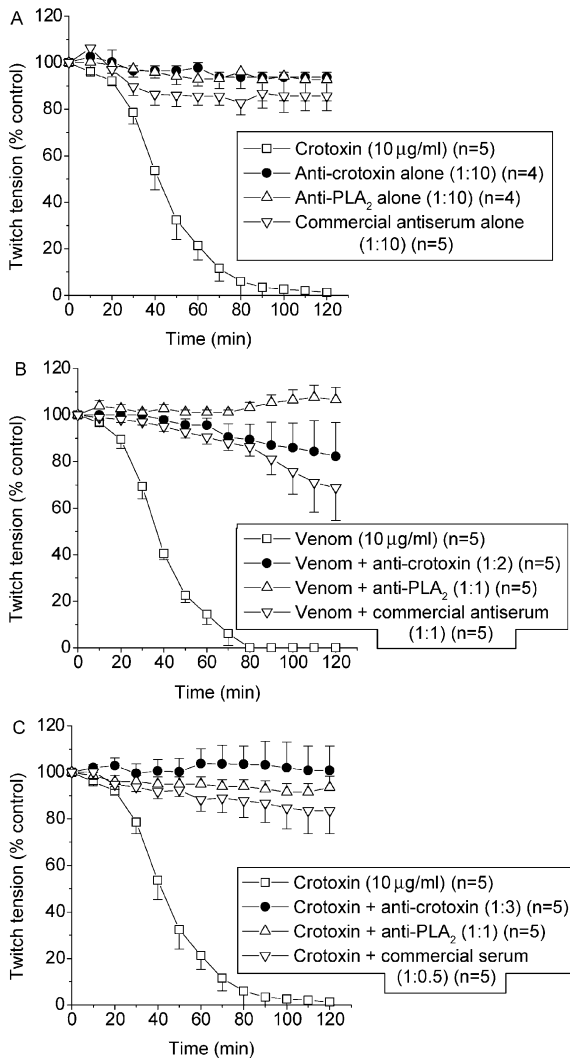

Fig. 3. Neutralization by anti-crotoxin, anti-PLA_2 and commercial anti-sera of the neuromuscular blockade caused by *C. d. cascavella* venom and crotoxin in mouse phrenic nerve–diaphragm preparations. The anti-sera alone caused no significant neuromuscular blockade (A). Panels (B) and (C) show the protection by anti-sera against the neuromuscular blockade induced by *C. d. cascavella* venom and crotoxin, respectively. Each point is the mean ± SEM of 3–5 experiments. In the anti-serum groups in (B) and (C), all time points from 20 min onwards were significantly different ($P < 0.05$) from venom or crotoxin alone.

lower than those indicated (data not shown). Fig. 3C shows the neuromuscular responses to crotoxin preincubated with anti-crotoxin, anti-PLA_2 or commercial crotalic anti-venom. The best protection by anti-crotoxin serum was obtained at a toxin:anti-serum ratio $\geq$1:3 ($P < 0.05$, for $t_{20-120\ min}$). Anti-PLA_2 serum protected against the neuromuscular blockade at a crotoxin:anti-serum ratio $\geq$1:1 ($P < 0.05$, for $t_{20-120\ min}$). A protective effect by commercial crotalic anti-venom was observed at a crotoxin:anti-venom ratio $\geq$1:0.5 ($P < 0.05$, for $t_{40-120\ min}$); there was no protection by any of the sera at lower ratios (data not shown).

3.3.2. Chick biventer cervicis preparation

None of the anti-sera alone had any significant effect on the basal neuromuscular responses of chick biventer cervicis preparations (Fig. 4A). Fig. 4B shows the neutralizing

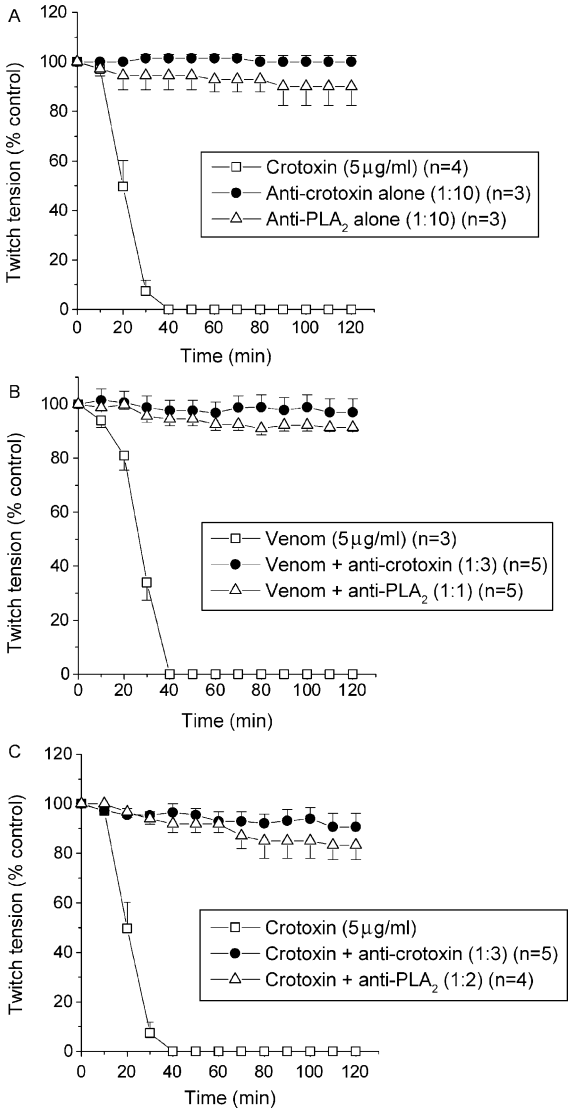

Fig. 4. Neutralization by anti-crotoxin and anti-PLA_2 sera of the neuromuscular blockade caused by *C. d. cascavella* venom and crotoxin in chick biventer cervicis preparations. The sera alone caused no significant neuromuscular blockade (A). Panels (B) and (C) show the protection by anti-crotoxin and anti-PLA_2 sera against the neuromuscular blockade induced by *C. d. cascavella* venom and crotoxin, respectively. Each point is the mean ± SEM of 3–5 experiments. In the anti-serum groups in (B) and (C), all time points from 20 min onwards were significantly different ($P < 0.05$) from venom or crotoxin alone.

capacity of anti-crotoxin and anti-PLA_2 anti-sera against the neuromuscular blockade produced by *C. d. cascavella* venom. Anti-serum to crotoxin protected against the neuromuscular blockade at a venom:anti-serum ratio $\geq$1:3 ($P < 0.05$, $t_{20-120\ min}$), while anti-serum to PLA_2 protected at a venom:anti-serum ratio $\geq$ 1:1 ($P < 0.05$, $t_{20-120\ min}$). At lower proportions, these anti-sera offered no significant protection (data not shown). Fig. 4C shows the response to crotoxin from *C. d. cascavella* venom in the presence of rabbit anti-sera (anti-crotoxin and anti-PLA_2). Anti-serum to crotoxin prevented the neuromuscular blockade at a crotoxin:anti-serum ratio $\geq$1:3 ($P < 0.001$, $t_{30-120\ min}$). For anti-PLA_2 serum, significant protection against the blockade was obtained at a crotoxin:anti-serum ratio $\geq$1:1 for anti-PLA_2 serum ($P < 0.001$, $t_{30-120\ min}$); no protection was seen at lower ratios (data not shown). The efficacy of commercial anti-venom was not examined in this preparation.

4. Discussion

Crotalus durissus cascavella occurs in northeastern Brazil, where it is an important public health problem (Martins et al., 1998). The venom of this subspecies has a similar biochemical composition and biological activities to the more widely studied *C. d. terrificus* (Santoro et al., 1999), and several studies have shown that *C. d. cascavella* venom and its components can affect renal function (Martins et al., 1998, 2002, 2003; Oliveira et al., 2003), inflammatory responses (Camara et al., 2003) and platelet aggregation (Marlas, 1982, 1985; Marlas et al., 1983a,b). While relatively well-characterized biochemically, there has been no detailed immunological study of *C. d. cascavella* venom, although Santoro et al. (1999) showed that the immunoreactivity of this venom with commercial crotalic anti-venom in ELISA and immunoblotting was similar to that of *C. d. terrificus* and *C. d. collilineatus* venoms.

As shown here, ELISA confirmed the excellent reactivity of the anti-sera to crotoxin and PLA_2, with the highest titers being obtained with the corresponding antigen used in immunization. The quality of the rabbit anti-sera was also confirmed by their ability to totally protect against the neuromuscular blockade induced by *C. d. cascavella* venom and crotoxin in the two nerve–muscle preparations tested. Commercial crotalic anti-venom also efficiently neutralized the blockade induced by venom or crotoxin in phrenic nerve–diaphragm preparations. The slightly greater neutralizing capacity of this anti-venom compared to the rabbit anti-sera may be more apparent than real since commercial anti-venoms are often semi-purified, globulin-rich preparations, whereas rabbit anti-sera are generally used without further processing (Lomonte et al., 1987). In addition, venoms are complex mixtures of different antigens and a variety of potentially antigenic sites is presented to the immune system of the animal during immunization, in contrast to purified toxins, which are antigenically less diverse. Although venoms may present more antigenic sites, not all of these are necessarily associated with the active site of toxins (Freitas et al., 1990).

As reported elsewhere (Freitas et al., 1990; Oshima-Franco et al., 1999), anti-serum produced against crotoxin from *C. d. terrificus* venom neutralized the principal lethal effects of this venom. Our results with anti-sera to crotoxin and PLA_2 from *C. d. cascavella* venom extended these findings and showed very efficient neutralization of the neurotoxicity of *C. d. cascavella* venom and crotoxin. The neutralization of the neuromuscular blocking activity of the venom by anti-crotoxin serum was expected since crotoxin is the principal toxin in *C. d. cascavella* venom (Santoro et al., 1999; Beghini et al., 2000). These results support the observations of Freitas et al. (1990) that immunization with crotoxin and/or the inclusion of anti-crotoxin antibodies as a supplement in polyclonal anti-venoms should be considered in order to obtain hyperimmune serum with a high level of protection against the toxic effects of *C. d. terrificus* venom.

The molecular interactions involved in the neurotoxicity of multimeric neurotoxins such as crotoxin are more complex than those of single chain neurotoxins and, in the case of crotoxin, could involve several regions of the crotapotin and PLA_2 molecules (Choumet et al., 1998). Investigations with monoclonal and polyclonal antibodies have shown that antibodies directed against PLA_2 or crotapotin can neutralize the toxicity of *C. d. terrificus* venom and crotoxin (Dos Santos et al., 1988; Choumet et al., 1989, 1992, 1998). Our findings for *C. d. cascavella* agree with these studies since the anti-PLA_2 serum protected against the neuromuscular blockade caused by venom or crotoxin, and was slightly more efficient than the anti-crotoxin serum. This protection by anti-PLA_2 further confirms the central role of PLA_2 in the toxicity of crotoxin and supports work showing that the inactivation of PLA_2 activity with *p*-bromophenacyl bromide or the removal of calcium ions abolished the neurotoxicity of crotoxin (Marlas and Bon, 1982).

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Capítulo 3

"Ability of rabbit antiserum against crotapotin to neutralize the neurotoxic, myotoxic and phospholipase A_2 activities of crotoxin from *Crotalus durissus cascavella* snake venom "

ABILITY OF RABBIT ANTISERUM AGAINST CROTAPOTIN TO NEUTRALIZE THE NEUROTOXIC, MYOTOXIC AND PHOSPHOLIPASE A_2 ACTIVITIES OF CROTOXIN FROM *CROTALUS DURISSUS CASCAVELLA* SNAKE VENOM

Daniela G. Beghini[a], Maria Alice da Cruz-Höfling[b], Léa Rodrigues-Simioni[c], Maria Carolina Delatorre[b], José Camilo Novello[a], Stephen Hyslop[c], Sérgio Marangoni[a,*]

[a]*Departamento de Bioquímica and* [b]*Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, 13083-970, Campinas, SP, Brazil*

[c]*Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), CP 6111, 13083-970, Campinas, SP, Brazil*

Running title: Antiserum to crotapotin from *C. d. cascavella* venom

[*]Author for correspondence: Dr. Sérgio Marangoni, Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, 13083-970, Campinas, SP, Brazil. Tel.: +55-19-3788-6134; Fax: +55-19-3788-6129; *E-mail address:* marango@unicamp.br

Abstract

The toxicity of crotoxin, the major toxin in *Crotalus durissus terrificus* (South American rattlesnake) venom, is mediated by its basic phospholipase A_2 subunit which is non-covalently associated with an acidic, enzymatically inactive subunit (crotapotin). In this work, we examined the ability of rabbit antiserum raised against crotapotin purified from *Crotalus durissus cascavella* venom to neutralize the neurotoxicity and myotoxicity of this venom and crotoxin, and to inhibit the enzymatic activity of the crotoxin complex and PLA_2 alone. This neutralization was compared with the ability of antisera against crotoxin and PLA_2 to neutralize the same biological and enzymatic activities. Rabbit antiserum to crotapotin partially neutralized the neuromuscular blockade caused by venom and crotoxin in electrically stimulated mouse phrenic nerve-diaphragm preparations, without preventing the resulting morphological damage or inhibiting the PLA_2 activity. In contrast, rabbit antisera to PLA_2 and crotoxin effectively neutralized the neuromuscular and PLA_2 activities. The slight increase in PLA_2 activity seen after incubation with anti-crotapotin antiserum probably resulted from the release of PLA_2 from the crotoxin complex, with the enzyme having greater activity when dissociated from the complex. The partial neutralization of the neurotoxicity of crotoxin by the antiserum to crotapotin suggested that this antiserum may prevent the interaction of intact crotoxin with its acceptor by causing dissociation of the crotoxin complex through binding to crotapotin.

Keywords: Antiserum; Crotapotin; Crotoxin; Neuromuscular blockade; Neutralization; Phospholipase A_2

1. Introduction

Crotoxin, the major toxin in venom of the South American rattlesnakes *Crotalus durissus terrificus* and *Crotalus durissus cascavella*, is a potent phospholipase A_2 β-neurotoxin that is formed by the non-covalent association of an acidic, non-enzymatic, non-toxic subunit (component A or crotapotin), derived from a PLA_2-like precursor by proteolytic cleavage (Aird et al., 1985), and a basic, weakly toxic PLA_2 subunit (component B) that binds to various membranes. Crotoxin produces neuromuscular blockade (Vital Brazil, 1966) by inhibiting the release of acetylcholine from nerve terminals (Vital Brazil and Excell, 1971; Hawgood and Smith, 1977), but can also cause myonecrosis (Gopalakrishnakone and Hawgood, 1984; Beghini et al., 2004a).

The toxicity of crotoxin results from the synergistic action of the two subunits, with crotapotin acting as a chaperon that increases the specificity of binding of the PLA_2 at the neuromuscular junction, thereby enhancing the lethal potency of the toxin (Bon et al., 1979). The stability of the interaction between the PLA_2 and crotapotin is therefore a major determinant of the toxicity of crotoxin (Faure et al., 1993). Binding experiments have shown that the two subunits of crotoxin dissociate upon interaction of the complex with biological membranes, with the PLA_2 binding to membranes while crotapotin is released into solution (Delot and Bon, 1994). In the absence of crotapotin, the PLA_2 adsorbs to membranes in a non-saturable manner, whereas in the presence of crotapotin the PLA_2 binds to a limited number of high-affinity binding sites (Jeng et al., 1978; Bon et al., 1979).

Since crotoxin is the main toxin in these venoms and is responsible for most of the major clinical effects of envenoming (Azevedo-Marques et al., 1985, 2003; Fan and Cardoso, 1995), various studies have investigated the immunogenicity of the two subunits of crotoxin and the efficacy of antibodies directed against these subunits in neutralizing the toxicity of this toxin (Hanashiro et al., 1978; Nakazone et al., 1984; Kaiser and Middlebrook, 1988; Choumet

et al., 1989, 1992, 1998, 1999; Demangel et al., 2000). Most of the antibodies that neutralize crotoxin have been found to react with the PLA_2 subunit, blocking its enzymatic activity and preventing the binding of the toxin to its acceptor (Choumet et al., 1989, 1992). In agreement with this, polyclonal antibodies raised in rabbits against the basic subunit of crotoxin (PLA_2) from *C. d. cascavella* venom totally neutralized the neuromuscular blockade produced by the venom and crotoxin (Beghini et al., 2004a). Similarly, a monoclonal antibody directed against the non-toxic subunit (crotapotin) from *C. d. terrificus* venom was found to be effective in neutralizing the toxicity of crotoxin (Choumet et al., 1992, 1998).

Apart from the few studies mentioned above, there has been very little work on the production and characterization of anti-crotapotin antibodies and on their ability to neutralize biological activities such as the myotoxicity caused by crotoxin. In this study, we examined the ability of polyclonal antibodies raised in rabbits against crotapotin from *C. d. cascavella* venom to neutralize the myotoxicity and neurotoxicity of this venom and crotoxin, as well as the enzymatic and hemolytic activities of crotoxin and PLA_2. The neutralizing capacity of this anti-crotapotin antiserum was compared with that of rabbit antisera to crotoxin and PLA_2.

2. Materials and methods

2.1. Venom and toxins

Desiccated *C. d. cascavella* venom was purchased from a private serpentarium (Batatais, SP, Brazil). Crotoxin, PLA_2 and crotapotin were purified from this venom as described by Beghini et al. (2000).

2.2. Animals

Male Swiss white mice (26-32 g) were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). The mice were housed at 25°C on a 12 h light/dark cycle and had free access to food and water. Male New Zealand white rabbits (2-3 kg) were purchased from an established breeder (Granja de Coelhos Grota Azul, Paulínia, SP) and were housed individually with free access to food and water. The experiments described here were done within the guidelines established by the Brazilian College for Animal Experimentation (COBEA).

2.3. Antisera

Rabbit antisera against crotoxin (anti-crotoxin) and PLA_2 (anti-PLA_2) were produced as described elsewhere (Beghini et al., 2004a), and a similar procedure was used to obtain antiserum against crotapotin. Initially, rabbits were immunized by successive i.m. and i.d. inoculations of 750 μg of crotapotin per animal. The first injections were given i.m. and included Freund´s complete adjuvant (Difco Laboratories, Detroit, MI, USA) in a 1:1 ratio. The subsequent boosters were given i.d. with the toxin dissolved in 0.9% (w/v) NaCl. The antibody levels in the sera were monitored by gel immunodiffusion (Ouchterlony, 1949) and ELISA (Chávez-Olórtegui et al., 1993). Blood samples were collected from a marginal ear vein and stored at 4°C overnight. The serum was subsequently separated by centrifugation and stored in aliquots at -70°C. The protein concentrations of the antisera against crotapotin, crotoxin and PLA_2 was 37 mg/ml, 32.8 mg/ml and 32 mg/ml, respectively, as determined by the method of Bradford (1976).

2.4. Enzyme-linked immunosorbent assay (ELISA)

The immunoreactivity of the anti-crotapotin antiserum with crotapotin, PLA_2, crotoxin or venom (5 μg/100 μl/well) was assessed exactly as described elsewhere (Beghini et al., 2004a). The final absorbance of the reactions was read at 492 nm with a SpectraMax 340 multi-well plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Immunoblotting

Crotoxin (10 μg) and crotapotin (60 μg) were run on 15% polyacrylamide minigels in SDS-PAGE (Hoefer SE 260 Mighty Small™ system) at 100 V for 2 h and the proteins then transferred electrophoretically to nitrocellulose membranes (0.45 μm) in a TE-22 minitransfer tank (Hoefer-Pharmacia Biotech Inc., San Francisco, CA, USA) at 400 mA for 1 h. Subsequently, the membranes were blocked at room temperature for 2 h in a solution of 5% non-fat milk/0.05% Tween 20. After washing six times in Tris-buffered saline (TBS; 0.01 M Tris-HCl, 0.17 M NaCl, pH 7.6), the membranes were incubated overnight with rabbit antiserum against crotapotin (diluted 1:1,500 in TBS). After washing again with TBS, bound antibodies were detected by incubation with a goat anti-rabbit immunoglobulin G (whole molecule)-peroxidase conjugate (Sigma; 1:1,000 in TBS) for 2 h at room temperature with shaking. At the end of this period, the blots were washed, developed with 4-chloro-1-naphthol (Sigma; 0.03% in 0.05 M Tris-HCl, pH 7.6, containing 0.03% H_2O_2) and documented (Beghini et al., 2004a).

2.6. Mouse phrenic nerve-diaphragm muscle preparation

The phrenic nerve-diaphragm muscle preparations (Bülbring, 1946) were obtained from mice anesthetized with chloral hydrate (300 mg/kg, i.p.) and sacrificed by exsaguination.

The diaphragm was removed and mounted under a tension of 5 g in a 5 ml organ bath containing Tyrode solution (pH 7.4, 37°C) of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 11.1, aerated with 95%O_2 and 5%CO_2. Supramaximal stimuli (4x threshold, 0.1 Hz, 0.2 ms) delivered from a Grass S48 stimulator (Astro-Med Inc., W. Warwick, RI, USA) were applied to the nerve through bipolar electrodes. Isometric muscle tension was recorded by a force displacement transducer (Load Cell BG-10 GM, Kulite Semiconductor Products Inc., NJ, USA) coupled to a physiograph (Gould, Model RS 3400) via a Gould universal amplifier (both from Gould Inc., Recording Systems Division, Cleveland, OH, USA). The preparations were allowed to stabilize for at least 20 min before the addition of venom or crotoxin (10 μg/ml) or of a mixture of venom or crotoxin:antiserum (anti-crotapotin), preincubated for 30 min at 37°C. In the latter cases, the final venom or crotoxin concentrations were the same as for experiments without antiserum.

The venom:antiserum ratios used in these incubations were 1:1 (w/v) for commercial crotalic antivenom (n = 4), 1:2 (w/v) and 1:1 (w/v) for anti-crotoxin and anti-PLA_2 antisera, respectively (n = 4 each) (Beghini et al., 2004a), and 1:10 (w/v) for anti-crotapotin (n = 6). Similarly, the crotoxin:antiserum ratios were 1:0.5 (w/v) for commercial crotalic antivenom (n = 3), 1:3 (w/v) and 1:1 (w/v) for anti-crotoxin and anti-PLA_2 antisera, respectively (n = 4 each) (Beghini et al., 2004a), and 1:20 (w/v) for anti-crotapotin (n = 6).

2.7. Histological analysis

After incubation for 120 min with venom alone, venom + antiserum, crotoxin alone, or crotoxin + antiserum (n=3-6 each), the phrenic nerve-diaphragm preparations were rapidly removed from the bath and immersed in Bouin´s fixative for 24-48 h. After fixation, the tissues were dehydrated and embedded in Historesin (Leica Instr. Gmbh, Nubloch/Heidelberg).

Sections 2 μm thick (Leica RM 2035 microtome) were stained with 0.5% toluidine blue (Vetec, SP, Brazil) in 5% (w/v) borax (Quimesp, SP, Brazil) prior to examination by light microscopy. The morphological damage was quantified by counting the number of normal and damaged fibers in three non-overlapping, non-adjacent areas of each muscle section (two sections per mouse). The proportion of damaged fibers was expressed as a percentage of the total number of fibers counted (630 for n = 3 preparations, 840 for n = 4 and 1260 for n = 6).

2.8. Indirect hemolysis

The assay of indirect hemolytic activity (Gutiérrez et al., 1988) was used to quantify PLA_2 activity and to examine neutralizing capacity of various antisera. Briefly, 0.3 ml of packed rat erythrocytes washed four times with saline solution, 0.3 ml of egg yolk diluted 1:4 (v/v) with saline solution and 0.25 ml of 0.01 M $CaCl_2$ were added to 25 ml of 0.8% agarose dissolved in PBS, pH 8.1. The mixture was poured onto plastic plates and, after solidification of the agar, wells 3 mm in diameter were punched out and filled with 15 μl of PLA_2 or crotoxin solution. The plates were incubated at 37°C for 20 h and the diameters of the hemolytic halos were then measured. As controls, 15 μl of saline solution and 15 μl of antiserum alone were tested. The neutralizing capacity of the antisera was tested by incubating PLA_2 or crotoxin with anti-crotoxin, anti-PLA_2 or anti-crotapotin antiserum at a ratio of 1:1 (w/v) for 30 min at 37°C. Subsequently, 15 μl of each mixture containing 0.15 μg of crotoxin or PLA_2 was added to the wells and the plates were incubated for 30 h at 37 °C. The quantity of crotoxin or PLA_2 used corresponded to the amount of each protein that produced a hemolytic halo with 50% of the area of the maximum hemolytic halo (defined as HD_{50} or the hemolytic dose 50%). Indirect hemolytic activity was expressed as the diameter (in cm) of the halo obtained.

2.9. Statistical analysis

Each experimental protocol was repeated 3-5 times, and the results were expressed as the mean±S.E.M. ANOVA followed by the Bonferroni test was used for statistical comparison of the data. A value of $p<0.05$ indicated significance.

3. Results

3.1. Production of antisera

The antibody levels during immunization with crotapotin were monitored by double immunodiffusion until strong immunoprecipitin bands were consistently obtained (results not shown), at which point the rabbits were bled and the antibody titers of the sera determined by ELISA. Figure 1 shows the reactivity of crotapotin, PLA_2, crotoxin and *C. d. cascavella* venom with serial dilutions of the antiserum to crotapotin, the endpoint dilution of which was 1×10^{-5} with each antigen. The corresponding endpoint dilutions for the antisera to crotoxin and PLA_2 were similar (3×10^{-6}) (Beghini et al., 2004a).

3.2. Immunoblotting

Figure 2 shows that the antiserum to crotapotin (diluted 1:1,500) cross-reacted with crotoxin and crotapotin from *C. d. cascavella* in immunoblots, with a principal band being seen in each case.

3.3. Neutralization of the neuromuscular activity

Neuromuscular activity was assayed using a single concentration (10 μg/ml) of *C. d. cascavella* venom or crotoxin in mouse phrenic nerve-diaphragm preparations. This concentration was chosen because in preliminary experiments it produced neuromuscular blockade within a reasonable timescale. The times required for 50% blockade in the

preparations were 35.9 ± 1.1 min (n=5) and 41.2 ± 3.9 min (n=5) for venom and crotoxin, respectively, and were not significantly different.

Figure 3A shows the neutralization of venom-induced neuromuscular blockade by rabbit antiserum to crotapotin, which partially protected against the neuromuscular blockade by venom at a venom:antiserum ratio ≥ 1:10 ($P < 0.05$ from 10 min post-venom onwards up to 90 min, $t_{10\text{-}90\ min}$). Similarly, in Figure 3B, the antiserum partially protected against the crotoxin-induced neuromuscular blockade at a crotoxin:antiserum ratio ≥ 1:20 ($P < 0.05$, for $t_{20\text{-}70\ and\ 90\ min}$). The antiserum did not offer significant protection at ratios lower than those indicated (data not shown).

3.4. Neutralization of myotoxicity

Figure 4 shows the morphological appearance of fibers (cross-section) from an indirectly stimulated phrenic nerve-diaphragm preparation after a 120 min incubation in Tyrode solution (control). Most of the fibers were well-organized, although approximately one third (28.2 ± 7.3%) showed slight alterations. Figure 5 shows preparations incubated with venom alone (A), venom plus rabbit antiserum to crotoxin (B), PLA_2 (C) or crotapotin (D). There was extensive myonecrosis following incubation of the hemidiaphragm with the venom: 95.6 ± 2.5% (n=3) of the fibers showed the changes typical of damage by crotalid myotoxins (Fig. 5A). Pre-incubating venom with antiserum against crotoxin or crotapotin (1:2 and 1:10, v/v, respectively) reduced the percentage of myonecrosis to 53.2 ± 4% (n=4) and 66.7 ± 7.4% (n=6), respectively, which again was not significantly different from that caused by venom alone (Fig. 5B and D). However, when venom was pre-incubated with antiserum to PLA_2 (1:1, v/v), the myonecrosis was significantly reduced to 28.3 ± 12.8% (n= 4) ($P < 0.05$) compared to venom alone (Fig. 5C).

Figure 6 shows preparations incubated with crotoxin alone (A), crotoxin plus antiserum to crotoxin (B), PLA_2 (C) or crotapotin (D). The percentage of myonecrosis was 83.4 ± 10.8% (n=4) for crotoxin alone, 50.9 ± 15.1% (n=4) for crotoxin pre-incubated with anti-crotoxin (1:3, v/v), 47.6 ± 8.9% (n=4) for crotoxin pre-incubated with anti-PLA_2 (1:1, v/v) and 67.1 ± 8.2% (n=6) for crotoxin pre-incubated with anti-crotapotin (1:20, v/v). However, the reduction in the number of myonecrotic fibers caused by each of the antisera was not significant when compared to the damage caused by crotoxin alone. Note that the antiserum to crotapotin provided the least protection against the myotoxic effects of the venom and crotoxin.

3.5. Neutralization of PLA_2 activity

Figure 7 shows the hemolysis produced by crotoxin (A) and PLA_2 (B). The neutralizing capacity of the antisera was tested by incubating one HD_{50} of crotoxin or PLA_2 with antiserum to crotoxin, PLA_2 or crotapotin (1:1, v/v) for 30 min at 37°C prior to assaying the residual activity (Fig. 7A and B). The antiserum against crotapotin was unable to neutralize the enzymatic activity of crotoxin and PLA_2, whereas all of the other antisera totally neutralized this enzymatic activity.

4. Discussion

In this study, we produced antiserum to crotapotin by immunizing rabbits with crotapotin purified from crotoxin isolated from *C. d. cascavella* venom (Beghini et al., 2000). The titer of this antiserum against crotapotin was lower than those for rabbit antisera against crotoxin and PLA_2 (Beghini et al., 2004a), probably because crotapotin is a weak immunogen compared to other components of this venom (Kaiser et al., 1986). In addition, the reactivity of the antiserum with crotapotin was not markedly different from that seen when tested against

crotoxin or PLA_2, but was less than with venom. In contrast, antisera to crotoxin and PLA_2 reacted more with their respective antigens than with other molecules (Beghini et al., 2004a). The cross-reactivity between the antiserum to crotapotin and PLA_2 probably reflected the fact the crotapotin is derived from the proteolytic cleavage of a PLA_2-like precursor (Aird et al., 1985). Immunoblotting confirmed that the antiserum recognized purified crotapotin.

The antiserum to crotapotin only partially neutralized the neuromuscular blockade produced by crotoxin and the venom. In contrast, rabbit antisera to crotoxin and PLA_2 totally neutralize the neuromuscular blockade caused by crotoxin and venom in mouse phrenic nerve-diaphragm and chick biventer cervicis preparations (Beghini et al., 2004b), thus confirming the key role of PLA_2 in the toxicity of the crotoxin complex. Binding studies have shown that crotapotin is temporarily involved in the binding of crotoxin to its target site, which explains why the antiserum to crotapotin partially neutralized the neurotoxicity of crotoxin. These results reinforced the hypothesis by Choumet et al. (1998) that the neutralization by anti-crotapotin could involve dissociation of the crotoxin complex or the prevention of its interaction with the crotoxin receptor. The need for a larger volume of rabbit antiserum to partially neutralize crotoxin (1:20, v/v) compared to that required to neutralize the venom (1:10, v/v) probably reflected the lower content of crotoxin present in the venom compared to that of pure crotoxin used, or possibly that the immunocomplexation of anti-crotapotin occurred more easily in the venom than with purified crotoxin.

Histological analysis showed that the muscle fibers of preparations incubated with venom and crotoxin had alterations typical of myonecrosis. The limited protection offered by antiserum to crotapotin against the myotoxic action of the venom and crotoxin was expected based on the partial neutralization of the corresponding neuromuscular activity. However, rabbit antiserum to crotoxin, which efficiently protected against the neuromuscular blockade (Beghini et al., 2004a), provided no significant protection against venom- and crotoxin-

induced myonecrosis. In contrast, rabbit antiserum to PLA_2 prevented both the neuromuscular blockade and myotoxicity. This finding reinforces the view that the PLA_2 of crotoxin is the principal component responsible for the myotoxicity of *C. d. cascavella* venom. In *Bothrops* venoms, the predominant role of PLA_2 in myotoxicity has been demonstrated by using specific neutralizing antibodies or other inhibitory molecules (Lomonte et al., 2003). When these PLA_2 myotoxins are neutralized, most of the muscle damage they cause is prevented (Melo and Ownby, 1999; Moura-da Silva et al., 1990, 1991).

The antiserum to crotapotin did not inhibit the enzymatic activity of purified PLA_2 and crotoxin. This finding agrees with previous report with monoclonal antibody directed against crotapotin were able to neutralize the toxicity of *C. d. terrificus* venom and crotoxin without affecting the PLA_2 activity (Choumet et al., 1998). This discrepancy could be explained by suggesting that the immunocomplexation of the anti-crotapotin antibodies with crotapotin resulted in the dissociation of the crotoxin complex, with PLA_2 being released into solution, where it would be much less toxic than crotoxin, while still retaining its enzymatic activity. The slight increase in enzymatic activity seen in Figure 7A supports this hypothesis and suggests that PLA_2 without crotapotin has a higher enzymatic activity than when in the crotoxin complex.

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Figure legends

Fig. 1. ELISA reactivity of anti-crotapotin antiserum with venom, crotoxin, crotapotin and PLA_2 from *C. d. cascavella*. The plates were coated with antigen (5 μg/well) then incubated with antiserum at the dilutions indicated and finally with an appropriate IgG-peroxidase conjugate and substrate (OPD - orthophenilenodiamine). The resulting absorbances were read at 492 nm. Each point is the mean±S.E.M. of four determinations. In several cases, the error bars are smaller than the symbols.

Fig. 2. Immunoblot showing the reactivity of anti-crotapotin antiserum with crotoxin (1) and crotapotin (2) purified from *C. d. cascavella* venom. The molecular mass standards used were soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). The electrophoresis and transfer of proteins were done as described in Section 2.5. The amount of protein applied to each lane was 10 μg of crotoxin and 60 μg of crotapotin. The same results were obtained in two experiments.

Fig. 3. Neutralization by anti-crotapotin antiserum of the neuromuscular blockade caused by *C. d. cascavella* venom (A) and crotoxin (B) in mouse phrenic nerve-diaphragm preparations. The venom/toxin:anti-serum ratios used are shown in the respective panels. Each point is the mean±S.E.M. of 5-6 experiments.

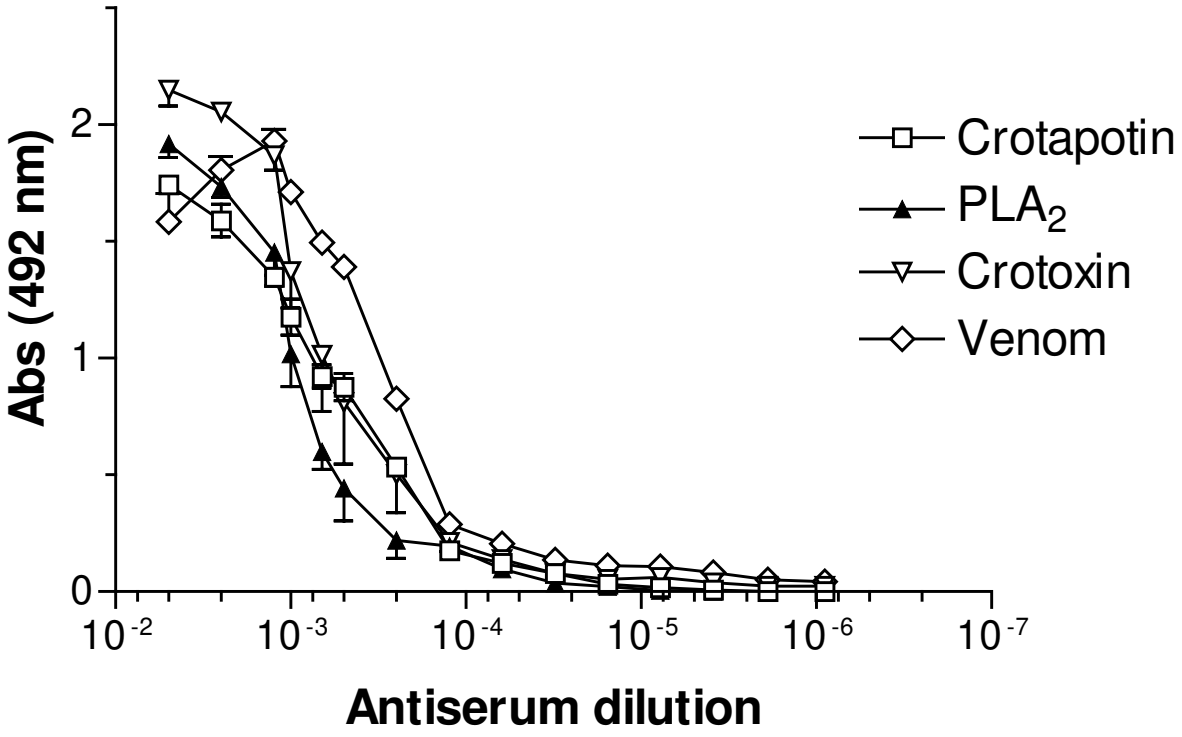
Fig. 4. Light micrograph of a control mouse phrenic nerve-diaphragm preparation (cross-section) showing the normal histological appearance of the muscle. Note the polygonal aspect of the fibers and peripheral nuclei (arrow). Toluidine blue staining. Bar = 15 μm.

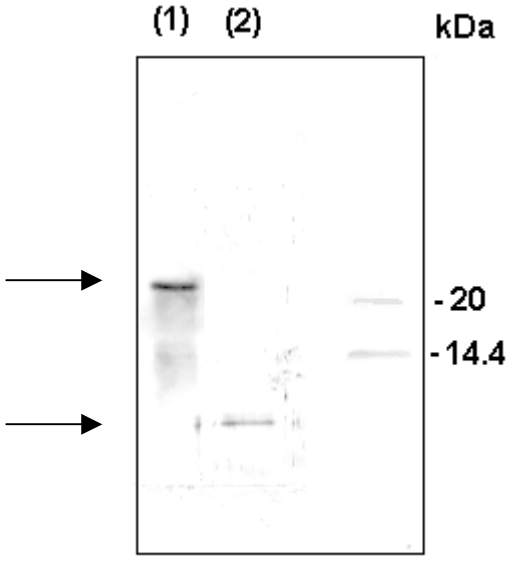
Fig. 5. Protection by antisera against muscle damage caused by *C. d. cascavella* venom. Panel A shows a cross-section of mouse diaphragm muscle fibers after incubation with *C. d. cascavella* venom (10 μg/ml) for 120 min. Note the presence of delta-type lesions, small clear vacuoles and ghost cells, the latter corresponding to areas of cytoplasm apparently devoid of myofibrils. Panels B-D show muscles treated with venom (10 μg/ml) that had been pre-incubated with with anti-crotoxin (1:2, w/v) or anti-PLA_2 (1:1, w/v) or anti-crotapotin (1:10, w/v) antiserum, respectively. Note that the anti-PLA_2 antiserum (panel C) was the most effective in preventing myonecrosis ($P < 0.05$) compared to venom alone. Anti-PLA_2 antiserum (panel C) caused only slight membrane disruption similar to that seen in control preparations (Fig. 4). Toluidine blue staining. Bars = 15 μm.

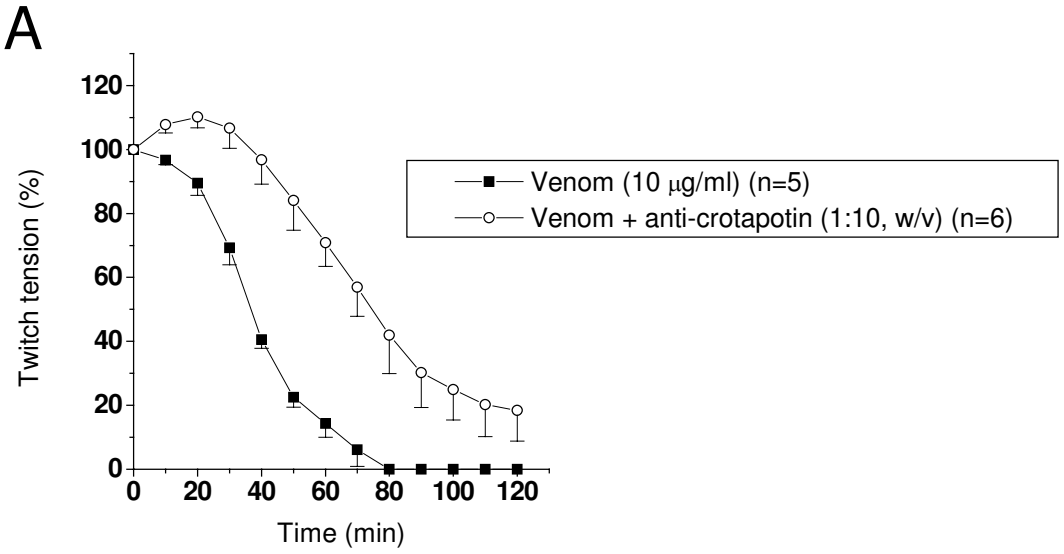
Fig. 6. Protection by antisera against muscle damage caused by crotoxin from *C. d. cascavella* venom. Panel A shows a cross-section of mouse diaphragm muscle fibers after incubation with crotoxin (10 μg/ml) for 120 min. Panels B-D show muscles treated with crotoxin (10 μg/ml) that had been pre-incubated with anti-crotoxin (1:3, w/v) or anti-PLA_2 (1:1, w/v) or anti-crotapotin (1:20, w/v) antiserum, respectively. Note that the antisera offered some protection against myonecrosis. The antiserum against crotapotin again provided the least protection (panel D). Toluidine blue staining. Bars = 15 μm.

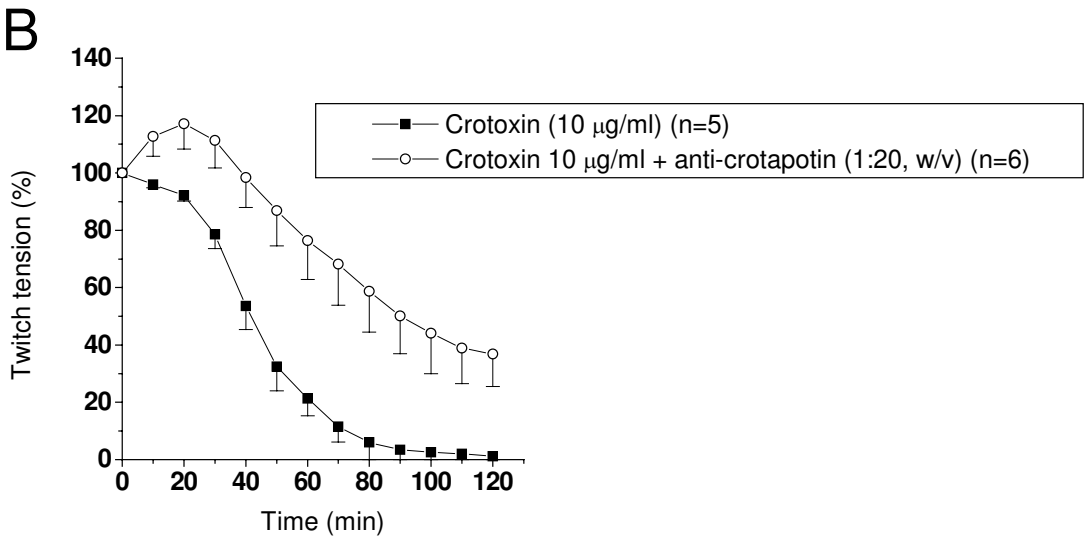
Fig. 7. Inhibition of the hemolytic activity of PLA_2 (A) and crotoxin (B) by anti-crotoxin (anti-CRTX), anti-PLA_2 (anti-PLA_2) and anti-crotapotin (anti-CRTP) antisera. Note that there

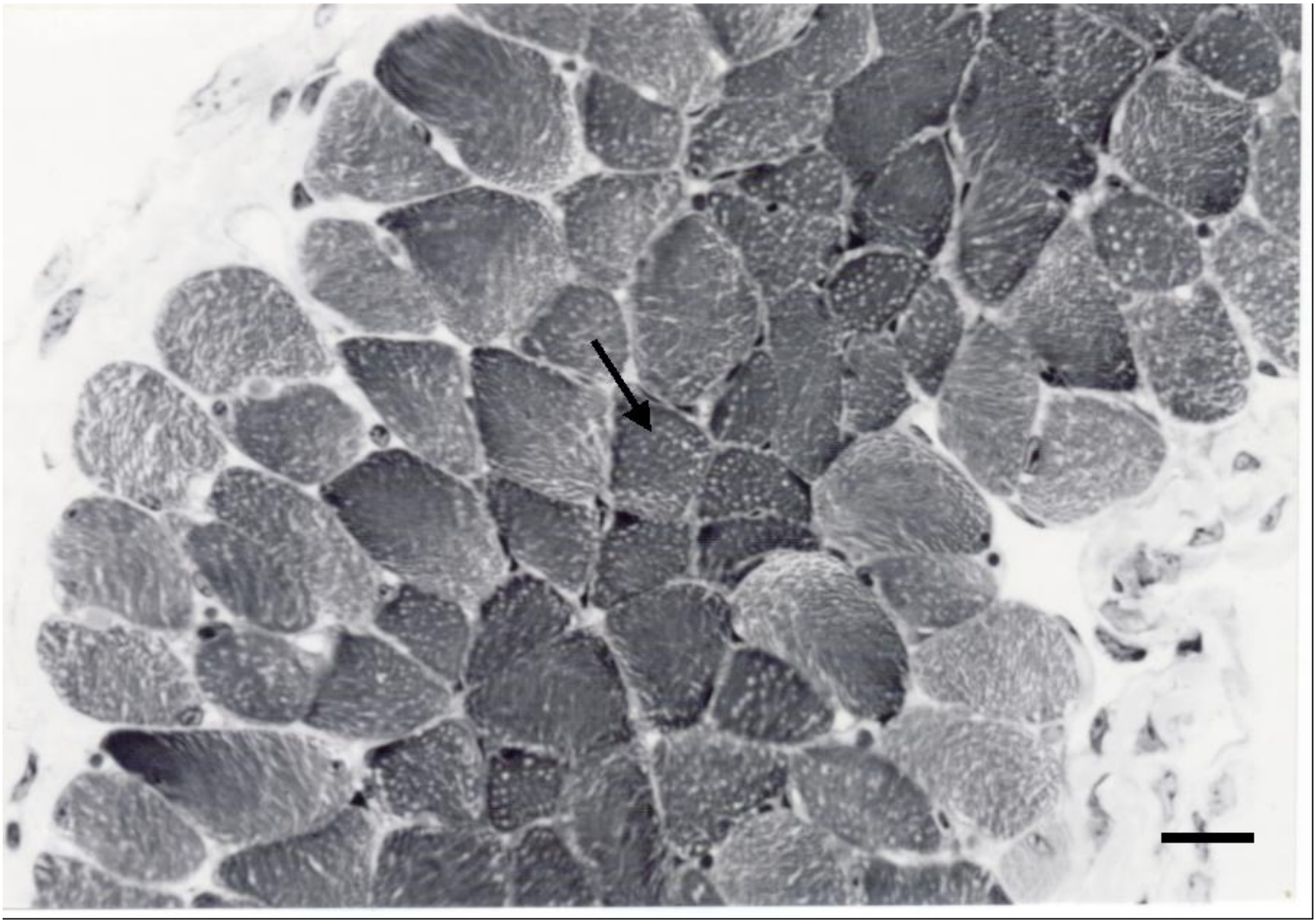
was no inhibition of hemolysis by the antiserum against crotapotin. $HD_{50\%}$ - amount of PLA_2 (0.15 μg) and crotoxin (0.15 μg) that caused 50% hemolysis. The crotoxin/PLA_2:antiserum ratio used was 1:1 in all cases. The columns represent the mean$\pm$S.E.M. of 6-10 experiments.

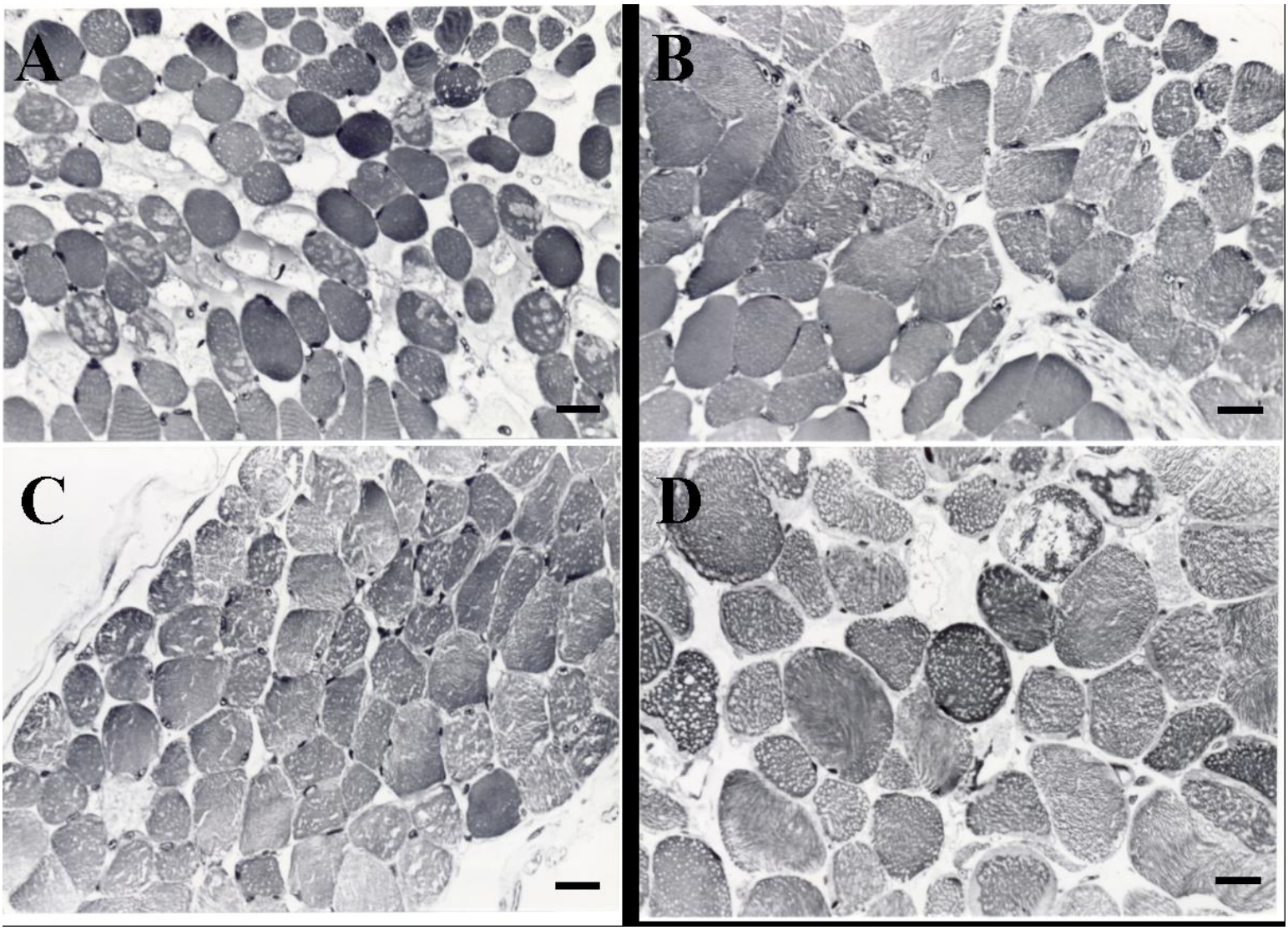

Abs (492 nm)
2
1
0
10-2
10-3
10-4
10-5
10-6
10-7
Antiserum dilution
Crotapotin
PLA2
Crotoxin
Venom

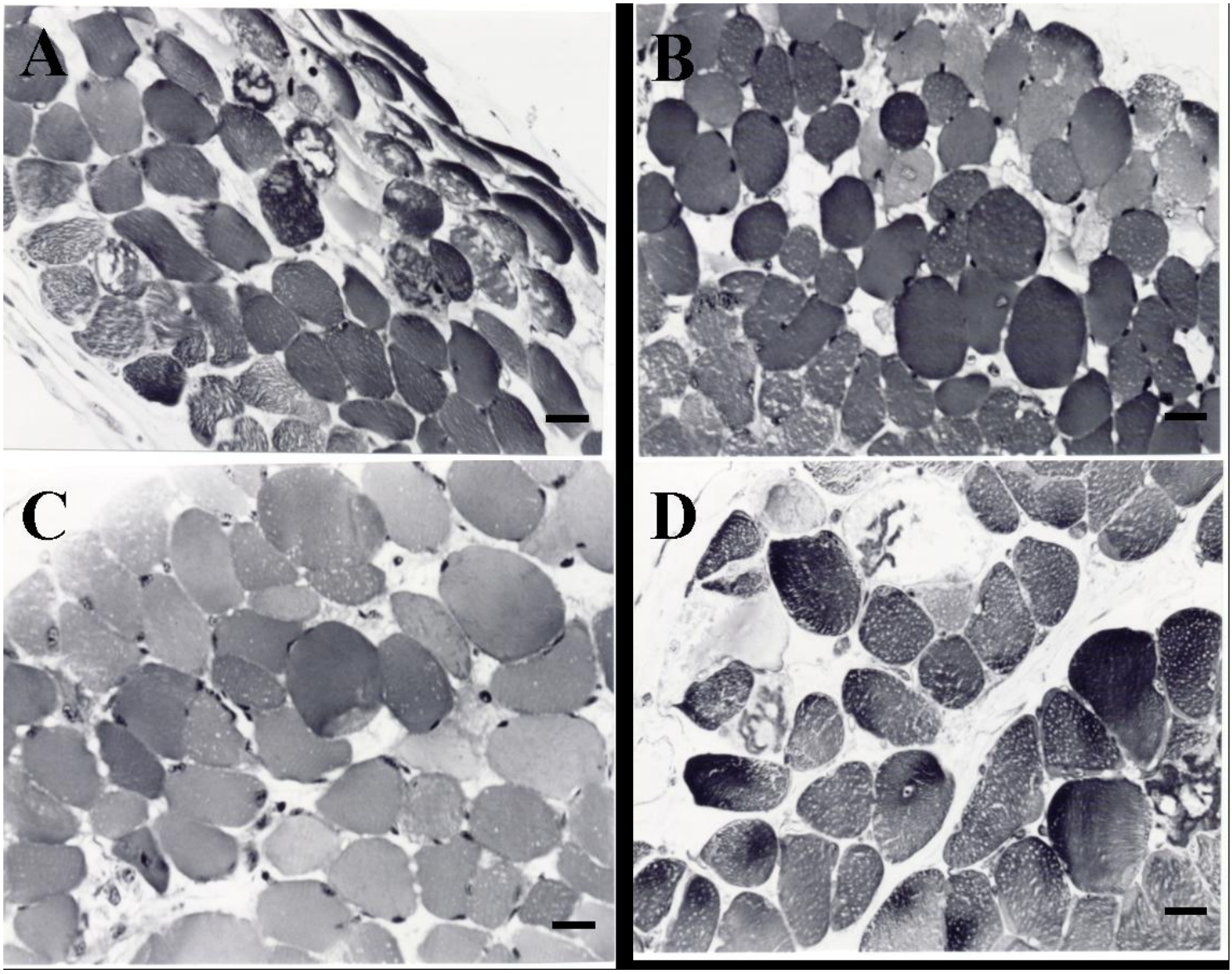

(1)
(2)
kDa
- 20
- 14.4

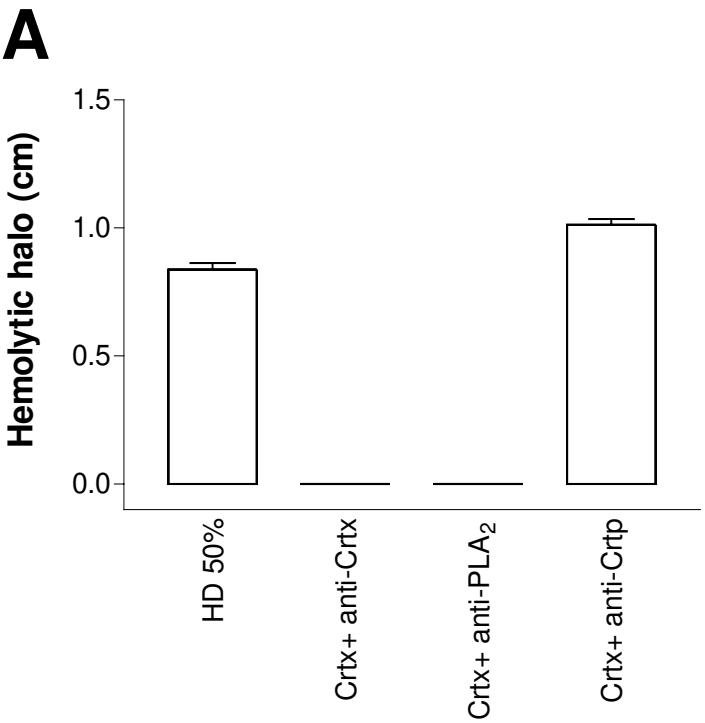

A
Twitch tension (%)
Time (min)
Venom (10 μg/ml) (n=5)
Venom + anti-crotapotin (1:10, w/v) (n=6)

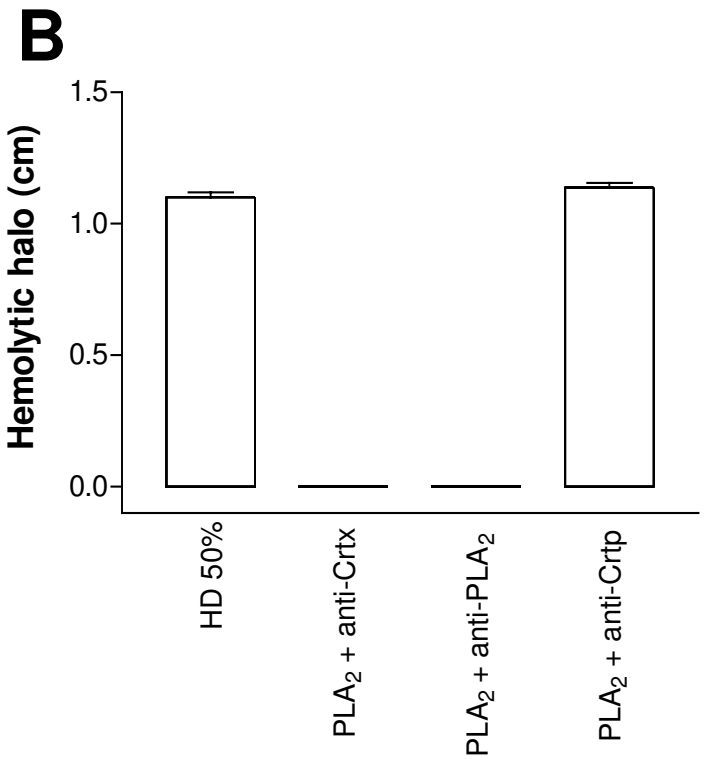

B
Twitch tension (%)
Time (min)
Crotoxin (10 μg/ml) (n=5)
Crotoxin 10 μg/ml + anti-crotapotin (1:20, w/v) (n=6)




A
B
C
D


A
B
C
D


A
Hemolytic halo (cm)
1.5
1.0
0.5
0.0
HD 50%
Crtx+ anti-Crtx
Crtx+ anti-PLA2
Crtx+ anti-Crtp


B
Hemolytic halo (cm)
1.5
1.0
0.5
0.0
HD 50%
PLA2 + anti-Crtx
PLA2 + anti-PLA2
PLA2 + anti-Crtp

Capítulo 4

"Cross-neutralization of the neurotoxicity of *Crotalus durissus terrificus* and *Bothrops jararacussu* venoms by antisera against crotoxin and phospholipase A_2 from *Crotalus durissus cascavella* venom"

Cross-neutralization of the neurotoxicity of *Crotalus durissus terrificus* and *Bothrops jararacussu* venoms by antisera against crotoxin and phospholipase A_2 from *Crotalus durissus cascavella* venom

Daniela G. Beghini[a], Maria Alice da Cruz-Höfling[b], Priscila Randazzo-Moura[c], Léa Rodrigues-Simioni[c], José Camilo Novello[a], Stephen Hyslop[c], Sérgio Marangoni[a,*]

[a]*Departamento de Bioquímica and* [b]*Departamento de Histologia e Embriologia, Instituto de Biologia, CP 6109, Universidade Estadual de Campinas (UNICAMP), CEP 13083-970, Campinas, SP, Brazil*

[b]*Departamento de Farmacologia, Faculdade de Ciências Médicas, CP 6111, Universidade Estadual de Campinas (UNICAMP), CEP 13083-970, Campinas, SP, Brazil*

Running title: Cross-reactivity of antisera to crotoxin and PLA_2

*Corresponding author: Tel.: +55-19-3788-6134; fax: +55-19-3788-6129.
E-mail address: marango@unicamp.br

Abstract

We have previously demonstrated that rabbit antisera raised against crotoxin and PLA_2 from *Crotalus durissus cascavella* venom neutralized the neurotoxicity of this venom and crotoxin. In this study, we examined the ability of these antisera to neutralize the neurotoxicity of *Crotalus durissus terrificus* and *Bothrops jararacussu* venoms and their major toxins, crotoxin and bothropstoxin-I (BthTX-I), respectively, in mouse isolated phrenic nerve-diaphragm preparations. Immunoblotting showed that antiserum to crotoxin from *C. d. cascavella* recognized crotoxin from *C. d. terrificus* and BthTX-I from *B. jararacussu*, while antiserum to PLA_2 from *C. d. cascavella* recognized PLA_2 from *C. d. terrificus* and BthTX-I from *B. jararacussu*. ELISA corroborated this cross-reactivity. Antiserum to crotoxin prevented the neuromuscular blockade caused by *C. d. terrificus* venom and its crotoxin at a venom/crotoxin:antiserum ratio of 1:3. Antiserum to PLA_2 also neutralized the neuromuscular blockade caused by *C. d. terrificus* venom or its crotoxin at venom or toxin:antiserum ratios of 1:3 and 1:1, respectively. The neuromuscular blockade caused by *B. jararacussu* venom and BthTX-I was also neutralized by the antisera to crotoxin and PLA_2 at a venom/toxin:antiserum ratio of 1:10 for both. Commercial equine antivenom raised against *C. d. terrificus* venom was effective in preventing the neuromuscular blockade typical of *B. jararacussu* venom (venom:antivenom ratio of 1:2), whereas for BthTX-I the ratio was 1:10. These results shown that antiserum produced against PLA_2, the major toxin in *C. durissus cascavella* venom, efficiently neutralized the neurotoxicity of *C. d. terrificus* and *B. jararacussu* venoms and their PLA_2 toxins.

Keywords: Antiserum; Cross-reactivity; Crotoxin; Neuromuscular blockade; Neutralization; Phospholipase A_2

1. Introduction

Venom phospholipases A_2 (E.C. 3.1.1.4) catalyze the hydrolysis of the *sn*-2-acyl bond of glycerophospholipids in a calcium-dependent fashion to release free fatty acids and lysophospholipids. These reaction products may exert biological activities directly or may be transformed into other active compounds (Dennis, 1994; Dessen, 2000) with hemostatic, cardiotoxic, convulsant, hemolytic, hypotensive, myotoxic and neurotoxic activities (Kini, 2003; Fuly et al., 2004). Various immunological studies have revealed considerable cross-reactivity among (Strong et al., 1994; Choumet et al., 1989; Mollier et al., 1990) and within (Middlebrook and Kaiser, 1989) the subclasses of venom PLA_2.

Crotoxin, the major toxin in the venoms of the South American rattlesnakes *Crotalus durissus terrificus, Crotalus durissus cascavella* and *Crotalus durissus collilineatus* (Slotta and Fraenkel-Conrat, 1938; Santoro et al., 1999), is a phospholipase A_2 (PLA_2) neurotoxin with potent neuromuscular blocking activity (Vital Brazil, 1966). Crotoxin consists of a basic, weakly toxic PLA_2 subunit and an acidic, non-toxic subunit (crotapotin) that is devoid of enzymatic activity (Hendon and Fraenkel-Conrat, 1971; Breithaupt, 1976). The similarities in the biological activities of *Crotalus d. terrificus*, *C. d. cascavella* and *C. d. collilineatus* venoms (Santoro et al., 1999) indicate that these venoms contain homologous proteins with similar structures, and this could be important for antigenic cross-reactivity and neutralization by heterologous antisera (Ménez, 1985).

Brazil (1903) was the first to report that crotalic antivenom was apparently more effective than bothropic antivenom in neutralizing the effects of *Bothrops jararacussu* venom, an observation which suggested that *Bothrops* snake venoms may contain components immunologically similar to toxins found in *C. d. terrificus* venom. More recently, Dos Santos et al. (1992) demonstrated that a mixture of bothropic and crotalic antivenoms provided the best neutralization of the biological activities of *B. jararacussu* venom. Vital Brazil (1966)

reported that *B. jararacussu* venom exerted a crotoxin-like action in rat phrenic nerve-diaphragm preparations, and various studies have since shown that this venom contains a variety of PLA_2 (Pereira et al., 1998; Ketelhut et al., 2003; Kashima et al., 2004), the best studied of which is bothropstoxin-I (BthTX-I), a Lys49 PLA_2 devoid of enzymatic activity (Cintra et al., 1993), but which causes neuromuscular blockade in avian and mammalian preparations (Heluany et al., 1992). Oshima-Franco et al., 2001 showed that the myotoxic and neurotoxic effects produced by BthTX-I was neutralized by antiserum against crotoxin and heparin, however these antiserum afforded greater protection than did heparin.

In view of the similarities among the venoms of Brazilian subspecies of *C. durissus* and the possible presence of similar toxins in crotalic and bothropic venoms discussed above, in this work, we used ELISA and immunoblotting to examine the ability of antisera raised in rabbits against crotoxin and PLA_2 from *C. d. cascavella* venom (Beghini et al., 2004a) to cross-react with the venoms of *C. d. terrificus* and *B. jararacussu* and their principal toxins, crotoxin and BthTX-I. We also assessed the ability of these antisera to neutralize the neurotoxicity of these venoms and toxins in mouse phrenic-nerve diaphragm preparations.

2. Materials and methods

2.1. Venoms and toxins

Desiccated *C. d. cascavella, C. d. terrificus* and *B. jararacussu* venoms were purchased from a private serpentarium (Batatais, SP, Brazil). Crotoxin and PLA_2 were purified from *C. d. cascavella* venom as described by Beghini et al. (2000). Crotoxin was also purified from *C. d. terrificus* venom as described elsewhere (Landucci et al., 1994), and BthTX-I was purified from *B. jararacussu* venom as described by Homsi-Brandeburgo et al. (1988).

2.2. Animals

Male Swiss white mice (26-32 g) were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). The mice were housed at 25°C on a 12 h light/dark cycle and had free access to food and water. Male chicks (4-8 days old, HY-LINE W36 lineage) were supplied by Granja Ito S/A (Campinas, SP, Brazil) and had free access to food and water. Male New Zealand white rabbits (2-3 kg) were purchased from an established breeder (Granja de Coelhos Grota Azul, Paulínia, SP) and housed individually with free access to food and water. The experiments described here were done within the guidelines established by the Brazilian College for Animal Experimentation (COBEA).

2.3. Antivenom

The commercial crotalic antivenom used was a semi-purified immunoglobulin-enriched solution produced by hyperimmunizing horses with *C. d. terrificus* venom (Instituto Vital Brazil, Rio de Janeiro, RJ, Brazil). This antivenom is distributed for use after peptic digestion and partial purification by ammonium sulfate precipitation, and contains 0.35% phenol as preservative.

2.4. Antisera

The immunization protocols used to produce antisera to crotoxin and PLA_2 and the procedures for monitoring the antibody titers are described by Beghini et al (2004a). The rabbits were immunized by successive i.m. and i.d. inoculations with 500 μg of crotoxin or 750 μg of PLA_2 per animal and the sera obtained by centrifugation after bleeding the rabbits were stored in aliquots at -70°C until used.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (96 wells) were coated overnight at 4°C with *C. d. cascavella, C. d. terrificus* or *B. jararacussu* venoms, crotoxin or BthTX-I (5 μg of venom or toxin/100 μl/well) in sodium bicarbonate buffer. The plates were washed and processed as described elsewhere (Beghini et al., 2004a), and the final absorbances were read at 492 nm with a SpectraMax 340 multi-well plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Purification of specific IgGs by affinity chromatography

Purified crotoxin and PLA_2 (Beghini et al., 2000) (30 mg each) were coupled separately to CNBr-Sepharose in 100 mM sodium bicarbonate, 500 mM NaCl, pH 8.3, according to the manufacturer's instructions (Hoefer-Pharmacia Biotech Inc., San Francisco, CA, USA). After preparation, the coupled Sepharose was loaded into two 6 ml columns (one for crotoxin and the other for PLA_2) and washed with phosphate-buffered saline (PBS), pH 7.4. Subsequently, 1 ml of anti-crotoxin and anti-PLA_2 antiserum was applied to the respective immunoaffinity column at a flow rate of approximately 10 ml/h. The columns were washed with PBS to remove unbound proteins and the absorbance of 1 ml fractions was read in a spectrophotometer at 280 nm. After the absorbance had returned to zero, bound proteins (specific IgGs) were eluted with 100 mM glycine-HCl, pH 2.8, and 1 ml fractions were collected in 100 μl of 1 M Tris-HCl, pH 9.0. The absorbance of the fractions was monitored as described above. This peak containing specific IgGs was lyophilized and used for immunoblotting (Chávez-Olórtegui et al., 1997). Due to the low yield, the affinity-purified IgG to crotoxin and PLA_2 were used only in immunoblotting to reduce nonspecific reactions and high backgrounds.

2.6. Immunoblotting

Crotoxin and PLA_2 from *C. d. terrificus* venom (10 or 15 μg each), BthTX-I and *B. jararacussu* venom (10 μg each) were run on 12.5% polyacrylamide minigels in SDS-PAGE (Hoefer SE 260 Mighty Small™ system) at 100 V for 2 h and the proteins then transferred electrophoretically to nitrocellulose membranes (0.45 μm) in a TE-22 minitransfer tank (Hoefer-Pharmacia Biotech Inc., San Francisco, CA, USA) at 400 mA for 1 h. The membranes were subsequently blocked, washed and incubated with primary antibodies (affinity-purified anti-crotoxin or anti-PLA_2 IgG, 0.001 μg/ml in TBS) and secondary antibodies (a goat anti-rabbit immunoglobulin G (whole molecule)-peroxidase conjugate; Sigma; diluted 1:1000 in TBS) as described elsewhere (Beghini et al., 2004a). The blots were finally developed with 0.03% 4-chloro-1-naphthol (Sigma) in 0.05 M Tris-HCl, pH 7.6, containing 0.03% H_2O_2) and documented.

2.7. Mouse phrenic nerve-diaphragm muscle preparation

The phrenic nerve and diaphragm (Bülbring, 1946) were obtained from mice anesthetized with chloral hydrate (300 mg/kg, i.p.) and sacrificed by exsaguination. The preparations were mounted in Tyrode solution (37°C) for electrical stimulation (stimuli: 4x threshold, 0.1 Hz, 0.2 ms) and the recording of contractile responses, as previously described (Beghini et al., 2004a). The preparations were allowed to stabilize for at least 20 min before the addition of *C. d. terrificus* venom or crotoxin (10 μg/ml each) or *B. jararacussu* venom or BthTX-I (50 μg/ml each). For the cross-neutralization tests, venom, crotoxin or BthTX-I was preincubated with the desired volume of rabbit antiserum or commercial antivenom for 30 min at 37°C, and then added to nerve-muscle preparations mounted as described above. In all

cases, the final concentrations of venom or toxin were the same as in the experiments without antiserum or antivenom.

2.8. Statistical analysis

Each experimental protocol was repeated 5-6 times, and the results were expressed as the mean±S.E.M. Statistical comparisons were done using ANOVA followed by the Bonferroni test. A value of $p<0.05$ indicated significance.

3. Results

3.1. Venom and toxin cross-reactivity as determined by ELISA

Figure 1A shows the reactivity of the anti-crotoxin antiserum against *C. d. cascavella, C. d. terrificus* and *B. jararacussu* venoms (5 μg/well). As expected, the greatest reactivity was seen with *C. d. cascavella* venom since the crotoxin used in the immunization was from this venom, whereas the least reactivity was seen with *B. jararacussu* venom.

Figure 1B shows the reactivity of antiserum to crotoxin from *C. d. cascavella* with crotoxins purified from *C. d. cascavella* and *C. d. terrificus* venoms (5 μg/well), and with BthTX-I (5 μg/well) from *B. jararacussu* venom. The crotoxins reacted best with the antisera, with that of *C. d. cascavella* being the most reactive, especially at antiserum dilutions $<10^{-4}$. Again, the least reactivity was obtained with BthTX-I.

The cross-reactivity of antiserum to PLA_2 with *C. d. cascavella*, *C. d. terrificus* and *B. jararacussu* venoms (5 μg/well) (Fig. 2A) was very similar to that seen with their respective toxins (Fig. 2B). Although BthTX-I (5 μg/well) showed greater cross-reactivity than the crotoxins at low antiserum dilutions, this reactivity decreased more rapidly than that of crotoxins at high serum dilutions.

3.2. Venom and toxin cross-reactivity as determined by immunoblotting

Affinity-purified IgGs to crotoxin and PLA_2 were used to reduce nonspecific reactions and high backgrounds in the membranes during immunoblotting. Figure 3A shows the cross-reactivity of *C. d. terrificus* venom and its crotoxin (lanes 1 and 2, respectively) and of *B. jararacussu* venom and BthTX-I (lanes 3 and 4, respectively) with purified IgGs raised against crotoxin from *C. d. cascavella* venom. These antibodies recognized crotoxin and PLA_2 from *C. d. terrificus* venom, as well as a band corresponding to BthTX-I (~15 kDa) from *B. jararacussu* venom.

Figure 3B shows the cross-reactivity of *C. d. terrificus* venom and its crotoxin (lanes 1 and 2, respectively) and of *B. jararacussu* venom and BthTX-I (lanes 3 and 4, respectively) with purified anti-PLA_2 IgG. The anti-PLA_2 IgG recognized the PLA_2 (~15 kDa) of *C. d. terrificus* crotoxin, as well as BthTX-I from *B. jararacussu* venom.

3.3. Cross-reactivity of antisera with venoms and toxins as determined by the neutralization of neurotoxicity

The venom/crotoxin:antiserum ratios used to examine the neutralization of neurotoxicity were chosen based on previous work (Oshima-Franco et al., 1999, 2001). Figure 4 shows the neutralization of the neurotoxicity of *C. d. terrificus* venom and its crotoxin by antisera to *C. d. cascavella* crotoxin and PLA_2 completely. The antisera showed an excellent neutralizing capacity against the blockade caused by *C. d. terrificus* venom at a venom:antiserum ratio of 1:3 ($p<0.05$, $t_{50\text{-}120}$) (Fig. 4A). The antisera to crotoxin and PLA_2 were also effective in neutralizing the neuromuscular blockade caused by crotoxin at ratios of 1:3 ($p<0.05$, $t_{40\text{-}120}$) and 1:1 ($p<0.05$, $t_{50\text{-}120}$), respectively (Fig. 4B). Smaller

venom/crotoxin:antiserum ratios (e.g. 1:0.5) did not provide effective neutralization (data not shown).

Figure 5 shows that the antisera to crotoxin and PLA_2 also effectively neutralized the neurotoxicity of *B. jararacussu* venom and BthTX-I, although this neutralization was not as complete as that seen for *C. d. terrificus* venom and crotoxin, despite the higher volumes of antisera used. Although the antiserum to crotoxin ($p<0.05$, t_{20-120}) and PLA_2 ($p<0.05$, t_{30-120}) prevented the neuromuscular blockade by *B. jararacussu* venom and BthTX-I at a venom/toxin:antiserum ratio of 1:10, BthTX-I was not as effectively neutralized as the venom. The need for a larger volume of antiserum to neutralize BthTX-I compared to that required neutralizing the venom probably reflected the lower content of BthTX-I present in the venom compared to that of pure BthTX-I used.

Commercial antivenom raised against *C. d. terrificus* venom neutralized the effects of *B. jararacussu* venom and BthTX-I to similar extents as the rabbit antisera but, unlike the later, also caused transient facilitation that peaked ~10 min after addition of the venom/toxin:antivenom mixture to the organ bath. No such effect was seen with the rabbit antisera. The anticrotalic commercial antivenom neutralized *B. jararacussu* venom in ratio of 1:2 ($p<0.05$, t_{10-120}) and protected against the neuromuscular blockade produced by BthTX-I in ratio of 1:10 ($p<0.05$, t_{20-120}). The blockade caused by BthTX-I was neutralized less effectively by the commercial crotalic antivenom than was the blockade caused by *B. jararacussu* venom, probably this reflect the lower content of BthTX-I present in the venom compared to that of pure BthTX-I used.

4. Discussion

The venom of *C. d. cascavella* has a similar biochemical composition and biological activities to the more widely studied *C. d. terrificus* (Santoro et al., 1999). Crotoxin is the main

neurotoxin in these venoms. We have demonstrated elsewhere (Beghini et al., 2004a) that antisera raised in rabbits against crotoxin and PLA_2 from *C. d. cascavella* venom had excellent neutralizing capacity against the neuromuscular blockade produced by this venom and its crotoxin. The results of the present study show that these same antisera are also effective in neutralizing the neurotoxicity of *C. d. terrificus* and *B. jararacussu* venoms and their corresponding toxins (crotoxin and BthTX-I, respectively).

The ELISA results confirmed the higher reactivity of the antiserum to crotoxin from *C. d. cascavella* used in the immunization protocol. The differences in the reactivity of this antiserum with crotoxins from the two venoms suggested that there were immunological differences between these two proteins, in agreement with variations in the biological activities of these same toxins (Beghini et al., 2004b). In contrast, there was little difference in the ELISA reactivities of PLA_2 from the two rattlesnake venoms, suggesting that the immunological differences between the crotoxins were attributable mainly to crotapotin, the acidic component of the complex. ELISA also revealed some immunological relatedness between the PLA_2 of *C. d. cascavella* and BthTX-I from *B. jararacussu* venom, a myotoxic Lys49 PLA_2 devoid of catalytic activity (Homsi-Brandeburgo et al., 1988). Immunoblotting using affinity-purified IgGs against crotoxin and PLA_2 generally confirmed with cross-reactivities with *C. d. terrificus* and *B. jararacussu* venoms and their principal toxins seen in ELISA. Overall, these results agree with other studies that have shown the existence of cross-reactivity among subclasses of venom PLA_2s (Choumet et al., 1989, 1992; Middlebrook and Kaiser, 1989).

The cross-reactivities seen in ELISA and immunoblotting correlated well with the cross-neutralization of the neurotoxicity, which suggested a key role for PLA_2 in the neuromuscular blockade. The antisera to crotoxin and PLA_2 effectively neutralized the neurotoxicity of the venom and crotoxin from *C. d. terrificus* in proportions similar to those used to neutralize the

venom and crotoxin from *C. d. cascavella* used in the immunization protocol (Beghini et al., 2004a). This cross-neutralization indicated the presence of identical epitopes involved in the neurotoxicity of the venoms and crotoxin from *C. d. cascavella* and *C. d. terrificus.*

Despite the weaker cross-reactivity of the antisera with *B. jararacussu* venom, the two antisera (against crotoxin and PLA_2) and commercial crotalic antivenom neutralized the neuromuscular blockade induced by this venom and BthTX-I, although in higher proportions than for rattlensnake venom and toxin. Oshima-Franco et al. (2001) have also shown that antiserum against crotoxin from *C. d. terrificus* venom neutralized the neuromuscular blockade and the myotoxic effects produced by BthTX-I, besides these antiserum was more effective in neutralizing the myotoxic effects of BthTX-I than was heparin. These results agree with the clinical observations of Brazil and Pestana (1909) regarding the efficiency of crotalic antivenom in the treatment of bites by *B. jararacussu.* Experimental and clinical evidences show that the addition of crotalic antivenom to bothropic homologous antivenom improved the results obtained in treatment of mice and patients bitten by *B. jararacussu* (Dos Santos et al., 1990, 1992).

The finding that antiserum produced against PLA_2 efficiently neutralized the neurotoxic activity of the venoms and toxins studied reinforces the central role of neurotoxic PLA_2 in disrupting neurotransmission. Freitas et al., (1990) suggest that immunization with crotoxin and/or addition of anti-crotoxin as a supplement to polyclonal antivenom should be considered in order to obtain a hyperimmune serum with a high level of protection against *C. d. terrificus* venom. However, the results in this work suggest that commercial antivenoms enriched in anti-PLA_2 antibodies could be useful for treating neurotoxicity in envenomed persons. In addition, the production of antivenoms against the main toxic component(s) of the venom of interest would reduce the amount of non-essential antibodies and proteins injected

during antivenom therapy and could therefore result in safer treatment (less risk of side effects such as anaphylactic shock).

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Figure legends

Fig. 1. ELISA reactivity of antiserum to crotoxin with the venoms of *C. d. cascavella*, *C. d. terrificus* and *B. jararacussu* (A) and their main toxins, crotoxin and BthTX-I (B). The plates were coated with antigen (5 μg/well) then incubated with antiserum at the dilutions indicated and finally with an appropriate IgG-peroxidase conjugate and substrate (OPD - orthophenilenodiamine). The resulting absorbances were read at 492 nm and each point is the mean±S.E.M. of four determinations, the error bars are smaller than the symbols. Note the immunological differences between the crotoxins from *C. d. terrificus* and *C. d. cascavella* venoms, and the lower reactivity of *B. jararacussu* venom and BthTX-I.

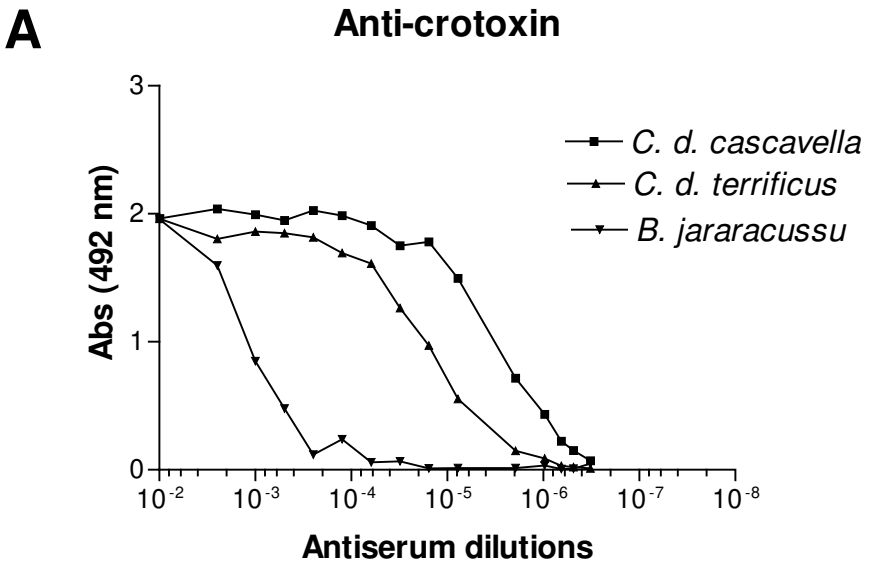
Fig. 2. ELISA reactivity of antiserum to PLA_2 with the venoms of *C. d. cascavella*, *C. d. terrificus* and *B. jararacussu* (A) and their main toxins, crotoxin and BthTX-I (B). The plates were coated with antigen (5 μg/well) then incubated with antiserum at the dilutions indicated and finally with an appropriate IgG-peroxidase conjugate and substrate (OPD). The resulting absorbances were read at 492 nm and each point is the mean±S.E.M. of four determinations, the error bars are smaller than the symbols. Note the immunological identity among the PLA_2 toxins (crotoxin and BthTX-I).

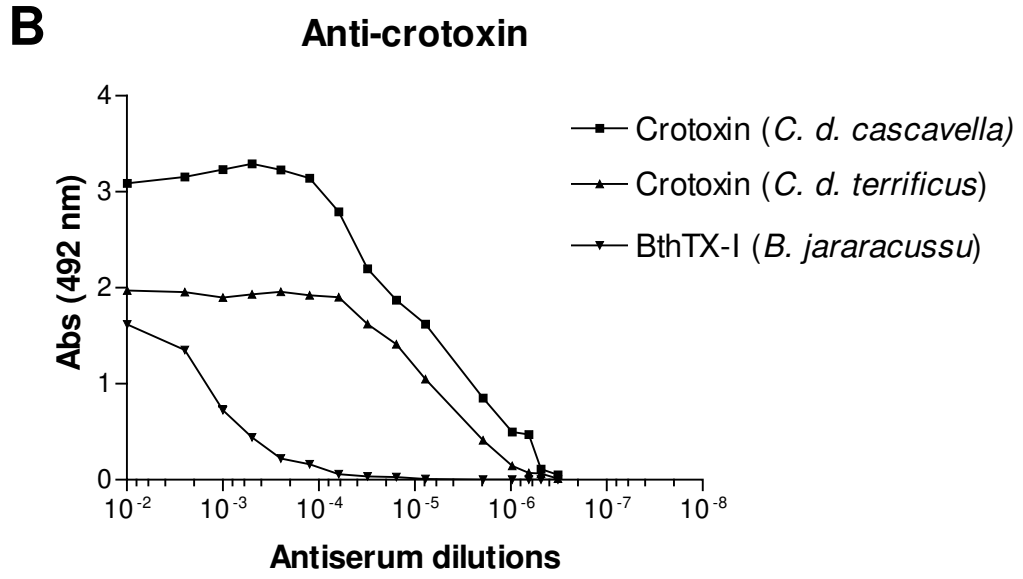
Fig. 3. Immunoblot showing the reactivity of affinity-purified anti-crotoxin (A) and anti-PLA_2 (B) IgG with *C. d. terrificus* venom (1), crotoxin (2), *B. jararacussu* venom (3) and BthTX-I (4). The molecular weight standards used were: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). The electrophoresis and transfer

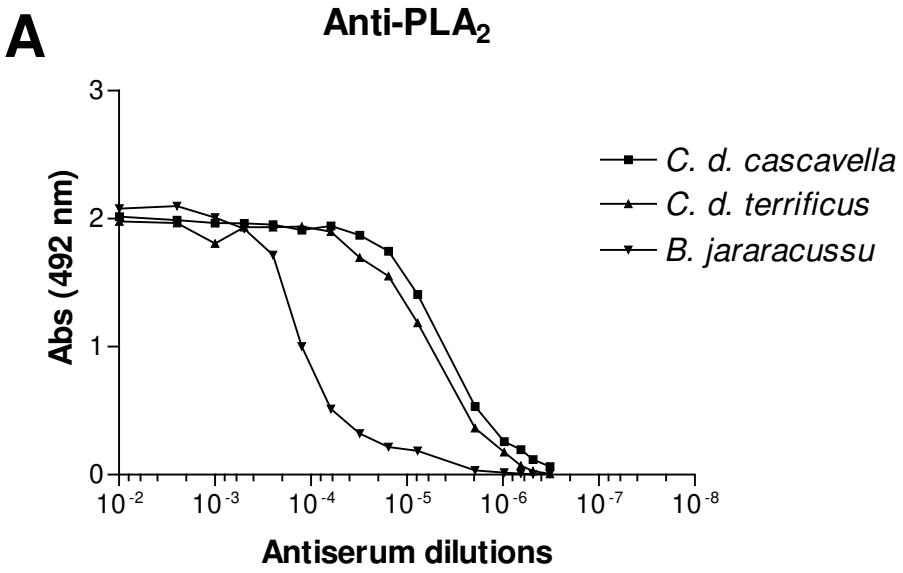
of proteins were done as described in Section 2.6. The amount of protein applied to each lane was 10 or 15 μg.

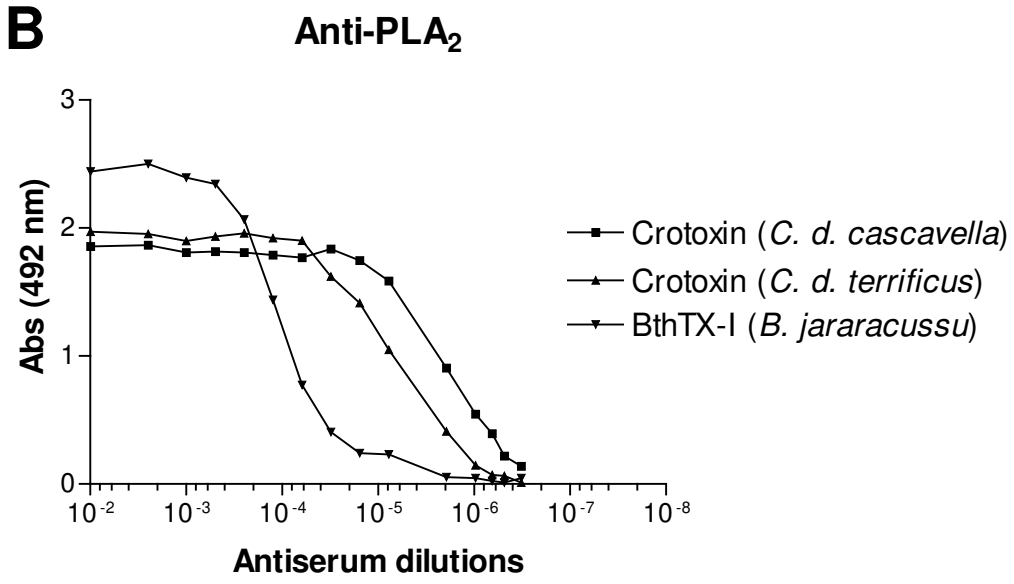
Fig. 4. Neutralization by antisera to crotoxin and PLA_2 from *C. d. cascavella* venom of the neuromuscular blockade caused by *C. d. terrificus* venom (A) and crotoxin (B) in mouse phrenic nerve-diaphragm preparations. Each point is the mean±S.E.M. of five experiments. In the antiserum groups in (A) and (B), all time points from 50 min onwards were significantly different ($p<0.05$) from venom or crotoxin alone.

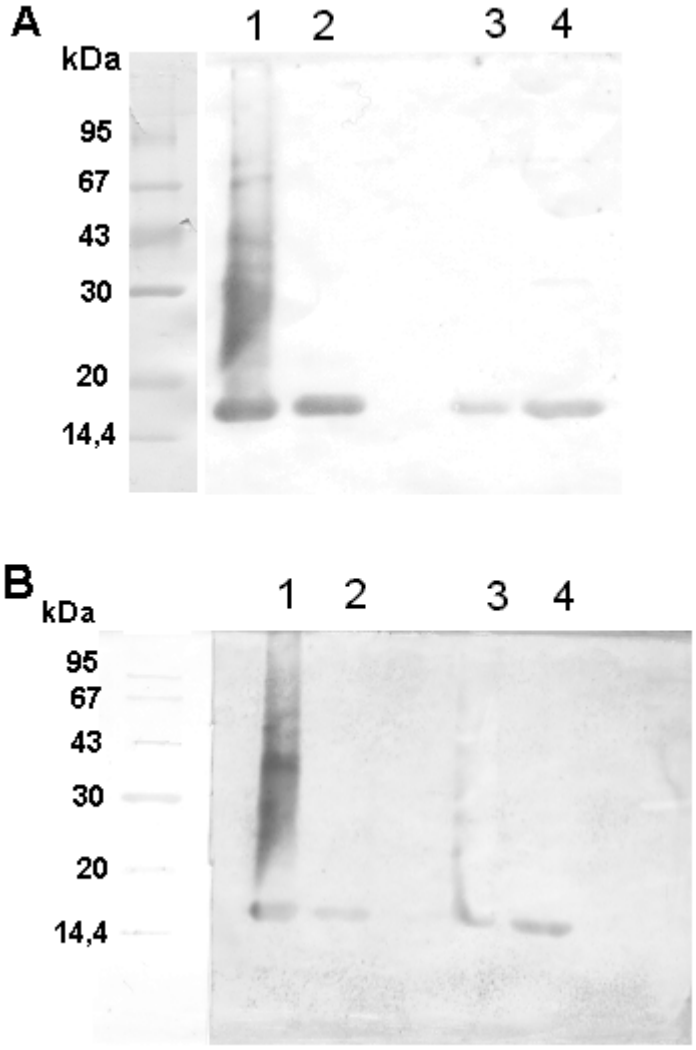
Fig. 5. Neutralization by antisera to crotoxin and PLA_2 from *C. d. cascavella* venom and of commercial crotalic antivenom against *C. d. terrificus* venom of the neuromuscular blockade caused by *B. jararacussu* venom (A) and BthTX-I (B) in mouse phrenic nerve-diaphragm preparations. Each point is the mean±S.E.M. of 5-6 experiments. In the antiserum groups in (A) and (B), all time points from 20 min onwards were significantly different ($p<0.05$) from venom or BthTX-I alone.

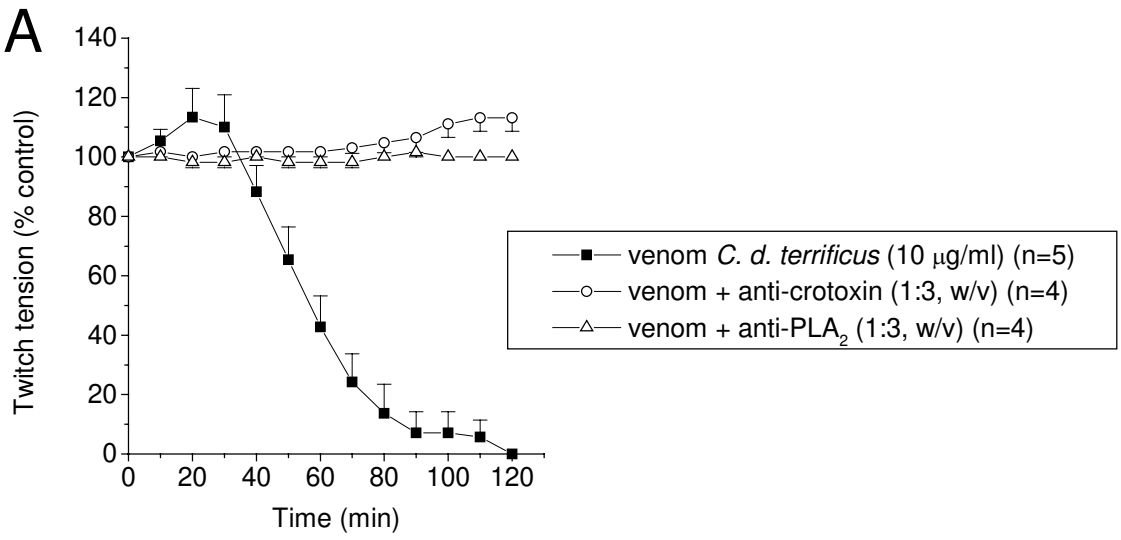

A
Anti-crotoxin
Abs (492 nm)
Antiserum dilutions
C. d. cascavella
C. d. terrificus
B. jararacussu

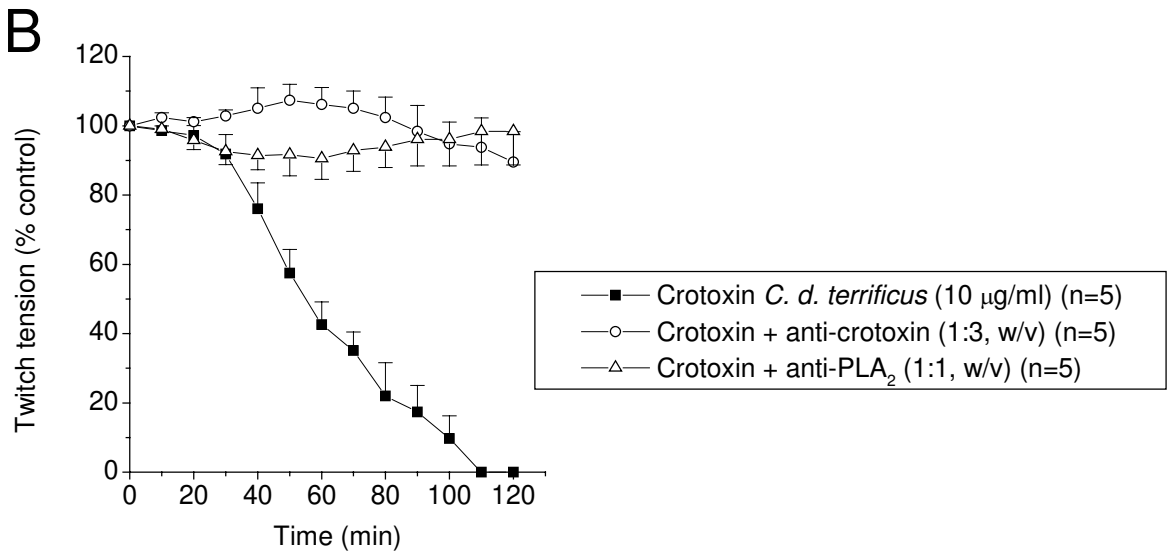

B
Anti-crotoxin
Abs (492 nm)
Antiserum dilutions
Crotoxin (C. d. cascavella)
Crotoxin (C. d. terrificus)
BthTX-I (B. jararacussu)

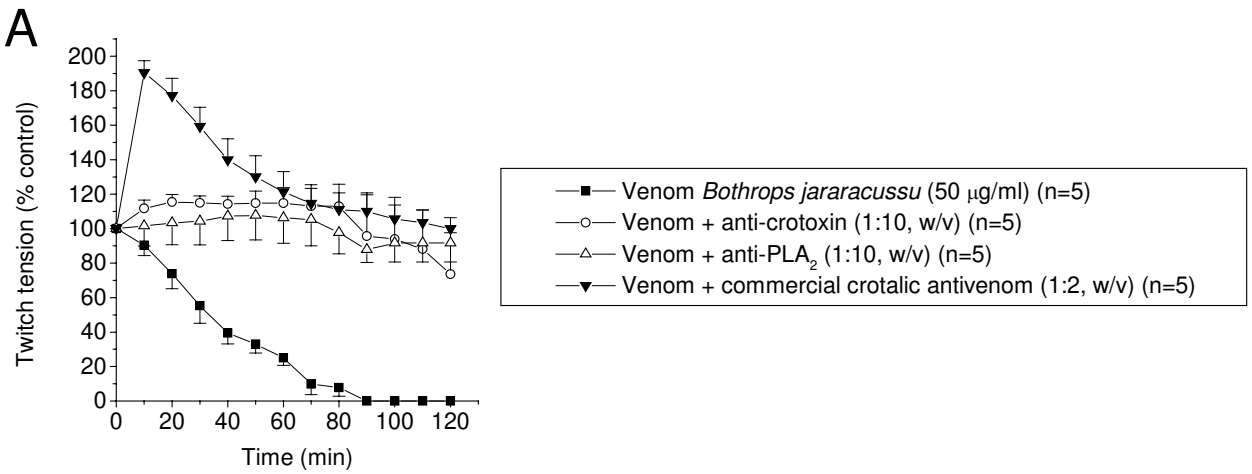

A
Anti-PLA2
Abs (492 nm)
Antiserum dilutions
C. d. cascavella
C. d. terrificus
B. jararacussu

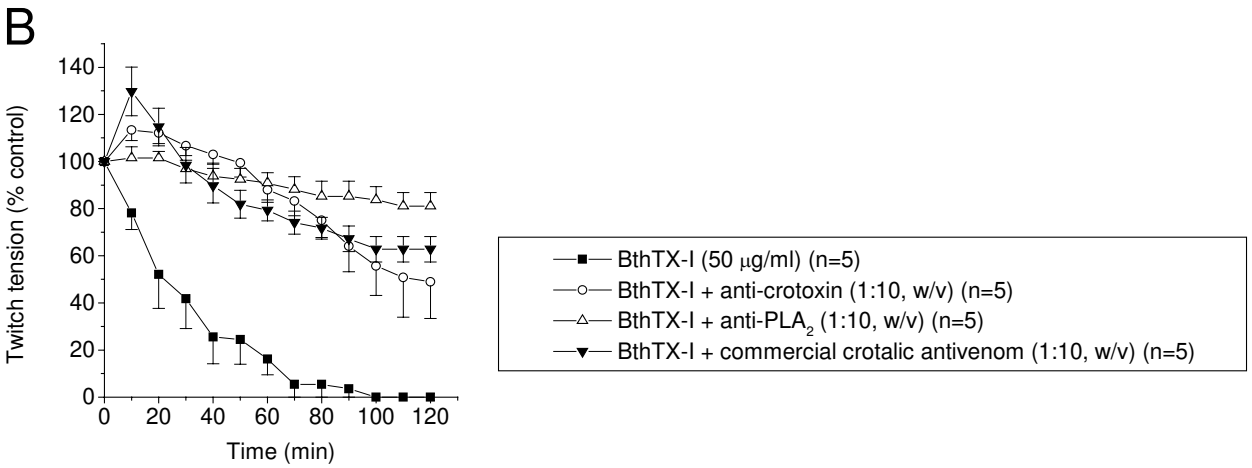

B
Anti-PLA2
Abs (492 nm)
Antiserum dilutions
Crotoxin (C. d. cascavella)
Crotoxin (C. d. terrificus)
BthTX-I (B. jararacussu)


A
1 2 3 4
kDa
95
67
43
30
20
14,4
B
1 2 3 4
kDa
95
67
43
30
20
14,4


A
Twitch tension (% control)
Time (min)
0
20
40
60
80
100
120
140
venom C. d. terrificus (10 μg/ml) (n=5)
venom + anti-crotoxin (1:3, w/v) (n=4)
venom + anti-PLA2 (1:3, w/v) (n=4)


B
Twitch tension (% control)
Time (min)
0
20
40
60
80
100
120
Crotoxin C. d. terrificus (10 μg/ml) (n=5)
Crotoxin + anti-crotoxin (1:3, w/v) (n=5)
Crotoxin + anti-PLA2 (1:1, w/v) (n=5)


A
Twitch tension (% control)
Time (min)
Venom *Bothrops jararacussu* (50 μg/ml) (n=5)
Venom + anti-crotoxin (1:10, w/v) (n=5)
Venom + anti-PLA2 (1:10, w/v) (n=5)
Venom + commercial crotalic antivenom (1:2, w/v) (n=5)


B
Twitch tension (% control)
Time (min)
BthTX-I (50 μg/ml) (n=5)
BthTX-I + anti-crotoxin (1:10, w/v) (n=5)
BthTX-I + anti-PLA2 (1:10, w/v) (n=5)
BthTX-I + commercial crotalic antivenom (1:10, w/v) (n=5)

Conclusões Gerais

CONCLUSÕES GERAIS

No capítulo 1 tivemos como conclusões principais:

- O veneno de *C. d. cascavella* e seu componente crotoxina possuem uma ação neurotóxica bastante potente e uma ação miotóxica presente somente em altas concentrações. Em concentrações tão baixas quanto 0,2 μg/ml de crotoxina ou veneno na preparação biventer cervicis de pintainho foi visto bloqueio neuromuscular completo e irreversível, o que evidenciou a potente neurotoxicidade.

- Além disso, foi mostrado que esse veneno possui uma ação predominantemente présináptica, pois após o bloqueio neuromuscular total as preparações continuaram respondendo com contratura à adição da acetilcolina e KCl.

- A análise morfológica também mostrou que o veneno total de *C. d. cascavella* é mais miotóxico do que a crotoxina. Outros componentes além da crotoxina podem estar contribuindo para essa miotoxicidade.

No capítulo 2:

- Os antissoros específicos produzidos em coelhos, anti-crotoxina e anti-PLA_2, apresentaram uma excelente qualidade evidenciada pelos testes de ELISA e immunoblotting. Além disso, apresentaram alta proteção do bloqueio neuromuscular induzido pelo veneno e crotoxina de *C. d. cascavella* nas preparações nervo-frênico diafragma de camundongo e biventer cervicis de pintainho.

- Nesse capítulo podemos também concluir que a produção de anticorpos contra o principal componente tóxico do veneno é suficiente para garantir a neutralização desse principal efeito tóxico.

- Nesse capítulo também evidenciamos a excelente capacidade neutralizante do bloqueio neuromuscular do antissoros anti-PLA_2, concluindo que a PLA_2 possui um papel central no mecanismo de neurotoxicidade. Também podemos sugerir que o sítio catalítico está decisivamente envolvido na ação neurotóxica.

No capítulo 3:

- ELISA mostrou que a crotapotina é um fraco imunógeno quando comparado aos outros componentes, crotoxina e PLA_2.
- O antissoro produzido contra a crotapotina parcialmente neutralizou a atividade neurotóxica do veneno e crotoxina. Esses resultados reforçam os achados de Choumet et al. (1998), que a neutralização por anti-crotapotina pode envolver a dissociação do complexo crotoxina ou a prevenção de sua interação com o receptor da crotoxina.
- A análise histológica mostrou que as fibras musculares da preparação nervo frênico diafragma de camundongo incubado com veneno ou crotoxina sofreram alterações típicas de mionecrose.
- Houve uma correlação positiva nos resultados obtidos pela técnica miográfica com a histológica quanto à fraca proteção oferecida pelo antissoro anti-crotapotina.
- O antissoro anti-PLA_2 produzido em coelho foi o mais eficiente em prevenir a mionecrose até mesmo frente ao antiveneno anticrotálico comercial. Isso reforça o já

visto por outros autores que a PLA_2 é o principal responsável pela miotoxicidade dos venenos brutos.

- O antissoro contra a crotapotina não inibiu a atividade enzimática da PLA_2 e crotoxina enquanto que os antissoros anti-crotoxina e anti-PLA_2 foram eficientes em inibir. Esse achado pode ser explicado através da sugestão que a imunocomplexação do anticorpo anti-crotapotina com a crotapotina resultaria na dissociação do complexo crotoxina com a PLA_2 sendo liberada na solução, a qual é menos tóxica do que a crotoxina, mas retém sua atividade enzimática.

No capítulo 4:

- ELISA mostrou reatividade dos antissoros anti-crotoxina e anti-PLA_2 com os venenos de *C. d. terrficus* e *Bothrops jararacussu* e suas principais toxinas. Entretanto, houve maior reatividade dos antissoros com veneno e crotoxina de *C. d. cascavella* usados na imunização. Immunoblotting confirmou a reatividade cruzada com os venenos e toxinas de *C. d. terrificus* e *B. jararacussu*.
- No ELISA também foi observada diferenças imunológicas entre as crotoxinas de *C. d. cascavella* e *C. d. terrificus*, e algumas semelhanças da PLA_2 com a BthTX-I de *B. jararacussu.*
- Os antissoros anti-crotoxina e anti-PLA_2 eficientemente neutralizaram a atividade neurotóxica do veneno e crotoxina de *C. d. terrificus* em proporções similares às usadas para neutralizar o veneno e crotoxina de *C. d. cascavella* usados na imunização.

- Anti-crotoxina, anti-PLA_2 e antissoro commercial anticrotálico neutralizaram o bloqueio neuromuscular induzido pelo veneno e BthTX-I de *B. jararacussu*. Entretanto, altas doses dos antissoros foram usadas na neutralização.
- Nesse capítulo foi mostrado que o antissoro produzido contra a PLA_2, principal componente tóxico em venenos de serpentes, eficientemente neutraliza a atividade neurotóxica desses venenos. Esse estudo, portanto, reforça os achados sobre o papel central da PLA_2 nos venenos com essa neurotoxina e nos leva a sugerir que o anti-PLA_2 poderia ser usado a fim de se obter um antissoro com alto nível de proteção contra a neurotoxicidade dos venenos.
- A produção de antissoros mais efetivos usando o componente mais tóxico do veneno deveria ser considerado, desde que há grandes vantagens em seu uso, porque anticorpos não-essenciais e proteínas poderiam ser removidos, e menos proteínas estranhas seriam usadas no tratamento, o que faria o antissoro ser mais seguro.

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