UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITULO DE BIOLOGIA - DEPARTAMENTO DE BIOQUÍMICA

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AÇÃO NEUROTÓXICA, MIOTÓXICA E CITOTÓXICA DO VENENO DE *Lachesis muta muta* (SURUCUCU) - CARACTERIZAÇÃO BIOQUÍMICA E BIOLÓGICA DE UMA PLA_2 (LmTX-I) PRESENTE NESTE VENENO

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. José Camillo Novello

CAMPINAS - SP
2006

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

Damico, Daniela Carla da Silva

D184a Ação neurotóxica, miotóxica e citotóxica do veneno de *Lachesis muta muta* (surucucu): caracterização bioquímica e biológica de uma PLA_2 (LmTX-I) presente neste veneno / Daniela Carla da Silva Damico. –-
Campinas, SP: [s.n.], 2006.

Orientador: José Camillo Novello.

Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. *Lachesis muta*. 2. Fosfolipase A_2. 3. Veneno. 4. Agentes neurotóxicos. I. Novello, José Camillo. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Título em inglês: Neurotoxic, myotoxic and cytotoxic action from the venom of the snake *Lachesis muta muta* (Bushmaster): biochemistry and biological characterization of a PLA_2 (LmTX-I) isolated from this venom.
Palavras-chave em inglês: *Lachesis muta*; Phospholipase A_2; Venom; Neurotoxic agents.
Área de concentração: Bioquímica.
Titulação: Doutora em Biologia Funcional e Molecular.
Banca examinadora: José Camillo Novello, Edson Antunes, Patrícia da Silva Melo, José Carlos Cogo, Daniela Diógenes de Carvalho.

Data da defesa: 02/03/2006.

Data da Defesa: 02/03/2006

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A Deus, pelo dom da vida.

Ao meu amor André, pelo apoio, compreensão, paciência e amor dispensados durante toda a realização deste trabalho. Obrigada por tudo, te amo!!

Aos meus pais, José Carlos e Adelina, que me deram à vida, e em especial a minha mãe, que sempre me apoiou e me incentivou em todas as circunstâncias da minha vida. Mã, obrigada por tudo, te amo.

As minhas queridas irmãs Paula e Karina, e aos meus sobrinhos Thiago, Matheus, Ruan e Maria Eduarda por serem simplesmente minhas irmãs e sobrinhos, que alegram minha vida.

AGRADECIMENTOS

Ao meu Orientador, Prof. Dr. José Camillo Novello pela confiança, incentivo e orientação durante a realização deste trabalho. Agradeço muito por ter acreditado na minha capacidade e ter me acolhido quando “cai de pára-quedas” como sua aluna.

Ao Prof. Dr. Sérgio Marangoni, que sempre apoiou e colaborou diretamente para a realização deste trabalho.

À Profa. Dra. Lea Rodrigues Simioni, do Departamento de Farmacologia, pela acolhida em seu laboratório, e por valiosas sugestões na realização deste trabalho.

À Profa. Dra. Maria Alice da Cruz-Höfling do Departamento de Histologia, pela acolhida em seu laboratório, pela valiosa ajuda, fundamental para a realização deste trabalho. Mach, muito obrigada!

À Profa. Dra. Carla B. Collares-Buzato, pela acolhida em seu laboratório, permitindo o aprendizado de novas técnicas e pela grande ajuda na elaboração do capítulo 4 desta tese.

Ao Prof. Dr. Stephen Hyslop, à Profa. Dra. Patrícia Melo da Silva, e à Profa. Dra. Carla B. Collares-Buzato, pela participação no exame de qualificação, onde puderam apresentar valiosas sugestões para revisão deste trabalho.

Aos membros da banca examinadora, pelas sugestões e correções feitas na tese.

A todos os amigos do Departamento de Bioquímica, especialmente do LAQUIP, pela convivência e troca de experiências durante estes anos.

A todos os amigos do Departamento de Farmacologia e Histologia, especialmente do laboratório da Profa. Dra. Léa Rodrigues Simioni, da Profa. Dra. Maria Alice da Cruz-Höfling e da Profa. Dra. Carla B. Collares-Buzato, pela acolhida e amizade.

Ao biólogo Gildo B. Leite, pela amizade e imprescindível apoio na realização de testes farmacológicos.

Ao Paulo Baldasso (Paulinho), pela amizade e apoio técnico no laboratório de Química de proteínas.

À Marta Beatriz Leonardo e à Rosana de Andrade Silva pela amizade e apoio técnico na elaboração das técnicas histológicas.

Aos docentes, funcionários e pós-graduandos do Instituto de Biologia da UNICAMP que direta e indiretamente, colaboraram para a realização deste trabalho.

A Capes, Coordenação de aperfeiçoamento de pessoal de nível superior, pelo imprescindível apoio financeiro.

À Faepex, Fundo de apoio ao ensino, à pesquisa e à extensão, pelo apoio financeiro durante congressos.

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RESUMO

A subespécie *Lachesis m. muta* vive em florestas tropicais úmidas de difícil acesso, dificultando sua captura e/ou manutenção em cativeiro (obtenção do veneno). Uma revisão de 20 casos de acidentes ofídicos com humanos ocorridos na Costa Rica, Guiana Francesa, Brasil, Colômbia e Venezuela, confidentemente atribuídos a este gênero de serpente, descrevem sintomas locais de dor, edema, equimose, coagulopatia, sintomas semelhantes aos observados no envenenamento bothrópico. Entretanto, náusea, cólica abdominal, vômito constante, diarréia e sudorese foram sintomas exclusivos, não reportados em vítimas de outros viperídeos do Brasil. Um dos objetivos deste trabalho foi estudar os efeitos neurotóxico e miotóxico do veneno de *L. m. muta* em preparações neuromusculares isoladas de ave (biventer cervicis de pintainho) e mamífero (nervo frênico diafragma de camundongo). Em baixa concentração (2 μg/ml), o veneno exibiu potente neurotoxicidade, entretanto, nesta concentração, nenhum efeito miotóxico significativo foi observado em preparação biventer cervicis de pintainho, a miotoxicidade foi observada somente a concentrações superiores, a partir de 10 μg/ml. Ficou claro que o veneno possui componentes ativos farmacologicamente que atuam na junção neuromuscular e nas fibras musculares, o qual a intensidade de ação depende da concentração do veneno e do tipo da preparação neuromuscular (ave ou mamífero) usada. O veneno bruto de *L. m. muta* também foi estudado quanto a sua ação citotóxica. O veneno mostrou um efeito citotóxico frente à célula tubular epitelial renal (MDCK), induziu a uma diminuição da viabilidade celular, alterou significantemente a resistência elétrica transepitelial através da monocamada e induziu alterações morfológicas e nucleares.

Foram purificadas duas novas toxinas (LmTX-I e LmTX-II) a partir do veneno total de *L. m. muta,* com um alto grau de pureza e homogeneidade molecular, sem perda da atividade biológica. Estas novas toxinas foram caracterizadas como isoformas de PLA_2s básicas Asp49, em função das características físico-químicas evidenciadas. Frente a diferentes concentrações de substrato, LmTX-I mostrou um comportamento tipo alostérico. Na ausência de Ca^{2+} (1 mM) e na presença de íons divalentes como Zn^{2+} e Cu^{2+}, a atividade PLA_2 foi inibida. Foi utilizada preparação biventer cervicis de pintainho para estudar a atividade neurotóxica e miotóxica *in vitro* da PLA_2 (LmTX-I). A toxina produziu um bloqueio irreversível da transmissão neuromuscular em concentrações baixas como 1 μg/ml. Entretanto, nesta concentração, nenhum efeito miotóxico significativo foi produzido pela toxina. O bloqueio neuromuscular não foi acompanhado da inibição da resposta contrátil à adição da acetilcolina (ACh), desta maneira, o bloqueio neuromuscular produzido pela LmTX-I pode ser atribuído preferencialmente por um efeito inibitório na liberação de ACh, sugerindo uma ação pré-sináptica da toxina. Com uma maior concentração (30 μg/ml), LmTX-I afetou a resposta ao KCl (30% de inibição), uma concentração no qual foi observado alterações morfológicas significativas (15% de fibras danificadas), como células com tamanhos heterogêneos, vacuolizadas, ou células com miofibrilas compactadas. Entretanto, nenhum efeito citotóxico significativo, como alteração morfológica e nuclear, redução da viabilidade celular ou indução da liberação de lactato desidrogenase, usando célula tubular epitelial renal (MDCK) e celular muscular esquelética (mioblastos e miotubos) foi observado.

ABSTRACT

The subspecies *Lachesis m. muta* lives in tropical forests of hard access, hindering its capture and/or maintenance in captivity (obtaining of the venom). A review on 20 case-reports that occurred in Costa Rica, French Guiana, Brazil, Colombia and Venezuela, of bites in humans, reliably attributed to this snake genus, described the local symptoms of pain, swelling, blistering, and mild coagulopathy, and as similar to those caused by snakebites of the genus *Bothrops* and other Latin American pit vipers genera, however, early nausea, abdominal colic, repeated vomiting, watery diarrhea and profuse sweating were distinctive symptoms, not reported in victims of other viperids. The venom of *L. m. muta* was studied with relationship to the neurotoxic and myotoxic effects in neuromuscular preparations isolated from avian (chick biventer cervicis muscle) and mammalian (mouse phrenic nerve-diaphragm muscle). The venom, in low concentrations (2 mg/ml) exhibited potent neurotoxicity, however, in this concentration, no significant myotoxic effect was observed in avian preparation, the myotoxicity was only observed to high concentrations, up to 10 mg/ml. It became clear that the venom has pharmacologically active components that act on neuromuscular junction and muscle fibers, whose intensity of action will depend on the venom concentration and on the type of nerve-muscle preparation (mouse or chick). The whole venom of *L. m. muta* also showed a cytotoxic effect in MDCK epithelial cell. The venom induced to a decrease of the cellular viability, altered significant the transepithelial electrical resistance through the monolayer and induced morphologic and nuclear alterations.

Through optimized methodologies of purification in HPLC, two new toxins were purified (LmTX-I and LmTX-II) from the venom of *L. m. muta*, with a high degree of purity and molecular homogeneity, without loss of the biological activity. These new toxins

were characterized as basic isoforms of PLA_2s Asp49, because they share several chemical and physical characteristics: molecular mass, retention time in re-purification on reverse phase HPLC, content of disulfide bonds, isoelectric point and primary structure. In the presence of different substratum concentrations, LmTX-I showed a behavior type allosteric under our experimental conditions. In the absence of Ca^{2+} (1 mM) and in the presence of divalent ions as Zn^{2+} and Cu^{2+}, the PLA_2 activity was inhibited. Avian muscle preparation was used to study *in vitro* neurotoxic and myotoxic activity of PLA_2 (LmTX-I). The toxin produced an irreversible blockade of the neuromuscular transmission in low concentrations as 1 mg/ml. However, in this concentration, the toxin produced no significant myotoxic effect. The complete neuromuscular blockade of the twitch tension was not accompanied of the inhibition of the response to ACh, this way, the neuromuscular blockade produced by the LmTX-I can be preferentially attributed by an inhibitor effect in the ACh release, suggesting a pre-synaptic action of the toxin. With a high concentration (30 mg/ml), LmTX-I affected the response to KCl (30% of inhibition) after the neuromuscular blockade have been completed, a concentration that was observed significant morphologic alterations (15% of damaged fibers), as cells with heterogeneous sizes, vacuolated, or cells with compacted myofibrils. However, any significant cytotoxic effect, as morphologic and nuclear alteration, reduction of the cellular viability or induction of the release of lactic dehydrogenase was observed using tubular epithelial renal and murine skeletal muscle cells.

INTRODUÇÃO

No Brasil, a maioria das serpentes venenosas pertence à família Viperidae (subfamília Crotalinae) e Elapidae. No Brasil, a família Viperidae é representada por 3 gêneros principais: *Crotalus* (cascavéis), *Bothrops* (jararacas) e *Lachesis* (surucucu). O envenenamento por cascavéis freqüentemente causa insuficiência respiratória aguda, relacionada à paralisia neuromuscular (Vital Brazil, 1972; Pellegrini Filho e Vital Brazil, 1976). O envenenamento por *Bothrops* causa principalmente efeitos locais, representados por edema, dor, hemorragia e mionecrose. Embora, distúrbios respiratórios sejam raros no envenenamento bothrópico, tal fenômeno foi reportado por Ribeiro et al. (1988), levantando a possibilidade do veneno bothrópico também exercer uma ação neurotóxica.

A filogeografia da serpente surucucu (gênero *Lachesis*) foi recentemente atualizada usando sequênciamento de DNA mitocondrial (Zamudio e Green, 1997). Três espécies e duas subespécies foram classificadas: *Lachesis stenophrys* (encontrada na costa atlântica da Costa Rica, Panamá e noroeste da América do Sul), *Lachesis melanocephala* (encontrada na costa pacífica do sudoeste da Costa Rica) e *Lachesis muta* (encontrada na floresta equatorial da Colômbia, Venezuela, Suriname, Guiana Francesa, Brasil, Equador, Peru e Bolívia) (Rosenthal et al., 2002). No Brasil encontramos as duas subespécies da *Lachesis muta*: *L. m. muta* (presente na bacia Amazônica) e *L. m. rhombeata* (Mata Atlântica do norte do Rio de Janeiro até Paraíba).

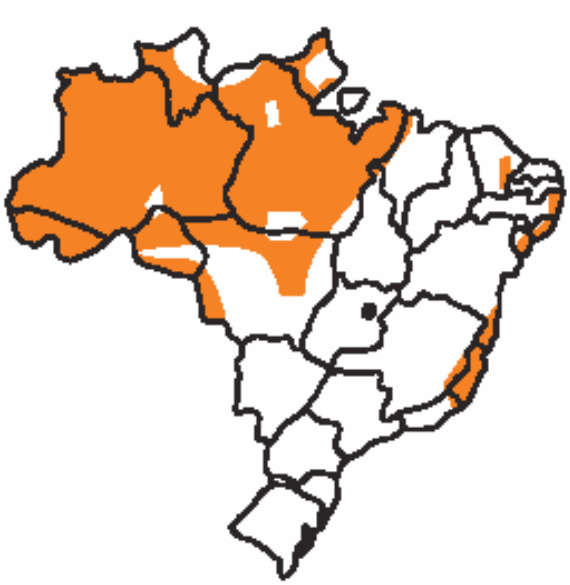


Figura 1. Distribuição da espécie *Lachesis muta* no Brasil (Fonte: Melgarejo, 2003).

Uma revisão de 20 casos de acidentes ofídicos com humanos que ocorreu na Costa Rica, Guiana Francesa, Brasil, Colômbia e Venezuela, confiantemente atribuídos a este gênero de serpente, descrevem sintomas locais de dor, edema, equimose, coagulopatia moderada, sintomas semelhantes aos observados no envenenamento bothrópico e em outros gêneros de viperídeos da América latina. Entretanto, náusea, cólica abdominal, vômito constante, diarréia e sudorese foram sintomas exclusivos (Jorge et al., 1997), não reportados em vítimas de outros crotalídeos do novo mundo. Entretanto, estes sintomas são remanescentes no envenenamento por víboras do velho mundo, como a víbora *Vipera berus* (Reid, 1976) e a “Palestine viper”, *Vipera palaestine* (Efrati e Reif, 1953).

Figura 2. Atitude de ameaça típica de *Lachesis m. muta* (Fonte: Melgarejo, 2003).

Na Venezuela, um paciente desenvolveu intensa sudorese, vômito, diarréia aquosa, hipersalivação, hipotensão, bradicardia e falha respiratória após 45 minutos de ser picado por um espécime jovem da surucucu (Torres et al., 1995). Este paciente também mostrou estrabismo divergente, disartria (dificuldade de articular as palavras) e disfagia (dificuldade de deglutir). Estes sintomas, provocados pelo envenenamento por *Lachesis*, constituem um tipo de "síndrome de envenenamento", não visto por vítimas de outros crotalídeos da América (Jorge et al., 1997), e é possivelmente causada por substâncias endógenas (autacóides), características de ativação do sistema nervoso autônomo parassimpático.

Epidemiologia

Acidentes humanos envolvendo serpentes do gênero *Lachesis* são raros e poucos casos têm sido documentados na literatura. A subespécie *L. m. muta* vive em

florestas tropicais úmidas de difícil acesso, o que explica em parte, a baixa incidência de casos de envenenamento com esta serpente.

No Brasil, constituem 1,4% do total de acidentes por serpentes peçonhentas, tendo sido notificados 939 acidentes provavelmente causados por esse gênero de serpente no período de 1990-1993, com 9 óbitos (0,9%) (Ministério da saúde, 1998).

Ações do veneno

O veneno laquético apresenta atividades fisiopatológicas semelhantes às do veneno bothrópico, ou seja, atividade coagulante, proteolítica, hemorrágica e inflamatória aguda. É relatada ainda, uma atividade cininogenase no veneno de *Lachesis muta,* que poderia explicar em parte, algumas alterações clínicas denominadas de "neurotóxicas" (Diniz e Oliveira, 1992). O quadro neurotóxico observado no envenenamento por *L. muta* é de ocorrência variável, e distingue o acidente laquético do bothrópico. Caracteriza-se, pela instalação precoce de hipotensão arterial grave (nos primeiro minutos após o acidente), e concomitantemente, descreve-se sudorese, náuseas, vômitos, cólicas abdominais, diarréia e bradicardia. A intensificação dessas alterações pode levar a choque, bradicardia grave e óbito (Jorge et al., 1997).

Também foi descrito no veneno uma atividade do tipo "trombina-like" (ação coagulante). Esta protease foi isolada inicialmente por Silva et al. (1985), e foi posteriormente seqüenciada e confirmada sua homologia com a giroxina crotálica (Magalhães et al., 1993; Aguiar et al., 1996). Além disso, foi identificada também uma fração ativadora de plasminogênio no veneno de *L. m. muta* (Sanchez et al., 2000).

Foram isoladas também uma lectina (lactose-específica), capaz de causar hemaglutinação e agregação plaquetária (Ogilvie et al., 1986) e uma fosfolipase A_2 ácida, com atividade inibidora da agregação plaquetária, bem como atividade miotóxica local in vivo e in vitro (Fuly et al., 1997; Fuly et al., 2000).

Até o momento foram isoladas duas metaloproteinases (LHF-I e LHF-II) com atividade hemorrágica no veneno de *L. m. muta* e que, provavelmente desempenham um papel importante nas anormalidades hemorrágicas descritas nos acidentes laquéticos. Além disso, atribui-se também a essas metaloproteinases atividade inflamatória local, formadora de edema e de degradação de componentes da matrix extracelular (Sanchez et al., 1987; Sanchez et al., 1995; Rucavado et al., 1999).

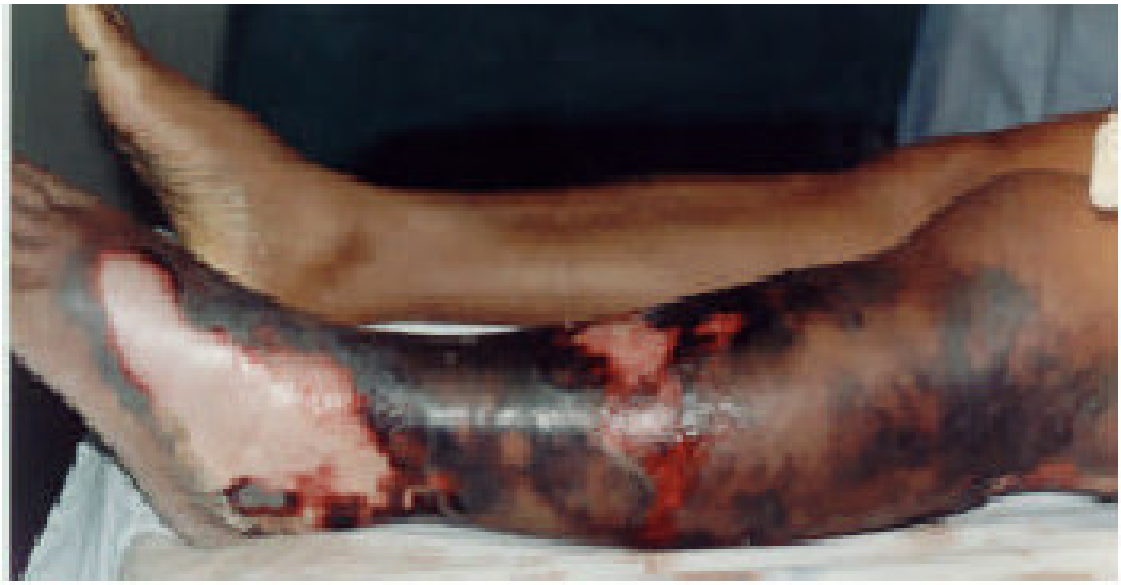


Figura 3. Necrose extensa da perna esquerda decorrente de acidente laquético grave (Foto: Araújo, M., 2003).

As fosfolipases (PLA_2)

Os venenos ofídicos são constituídos por diversas substâncias, cuja função é imobilizar, matar e digerir a presa. Alguns desses constituintes apresentam atividade PLA_2. Estas enzimas são divididas em várias classes, denominadas A_1, A_2, B, C e D, de acordo com o seu sítio de hidrólise. As fosfolipases A_2 catalisam a hidrólise da ligação acil éster na posição 2-sn dos fosfolipídios, liberando ácidos graxos e lisofosfolipídios (Kini, 2005). Alguns ácidos graxos livres podem atuar como mensageiros secundários ou podem estar envolvidos em outras reações, como exemplo, precursores de eicosanóides biologicamente ativos (Kini, 1997).

As PLA_2s extracelulares possuem baixo peso molecular (13 a 15 kDa), ocorrem abundantemente na natureza e requerem Ca^{2+} para desempenharem sua atividade catalítica. O suco pancreático dos mamíferos e os venenos de serpentes estão entre as fontes mais ricas em PLA_2s extracelulares (Verheij et al., 1981). Ao contrário das PLA_2s pancreáticas, as PLA_2s de venenos podem produzir potentes efeitos biológicos, tais como: neurotoxicidade (pré e pós-sináptica), miotoxicidade (local e sistêmica), cardiotoxicidade, efeito anticoagulante, indutor de agregação plaquetária, inibidor de agregação plaquetária, atividade hemolítica, convulsionante, hipotensiva, anti-hemorrágica, edematogênica e lesão de órgãos e tecidos (Kini, 1997). Ao comparar as seqüências de aminoácidos desses dois tipos de PLA_2s, observa-se que são poucas as substituições de resíduos de aminoácidos que convertem PLA_2s pancreáticas, desprovidas de toxicidade, em PLA_2s de venenos ofídicos altamente tóxicas, com especificidade para atuar em diferentes tecidos (Scott, 1997; Carredano et al., 1998).

Embora seus efeitos farmacológicos sejam diferentes, as diversas PLA_2s de venenos ofídicos apresentam alta homologia (cerca de 40 a 99%) entre suas

seqüências de aminoácidos. Por essa razão, não é fácil correlacionar às atividades farmacológicas de cada PLA_2 com suas diferenças estruturais. Assim, o estudo da relação entre estrutura e função desse grupo de proteínas é complexo e desafiante (Kini, 2005).

Várias novas PLA_2s tem sido identificadas, e diferem drasticamente das enzimas conhecidas. A evidência destas novas enzimas pode ser encontrada na recente reclassificação feita por Six e Dennis (2000). Até então, a classificação das PLA_2s se baseava na descrição feita por Dennis (1994), ou seja, em quatro grandes classes: I, II, III e IV, sendo que as PLA_2s das classes I, II, e III foram classificadas como enzimas extracelulares, caracterizadas por um alto conteúdo de pontes dissulfeto, em torno de 5 a 7 e por possuírem baixo peso molecular, em torno de 12 a 15 kDa. As PLA_2s da classe IV são constituídas pelas PLA_2s de alto peso molecular, de origem intracelular e que tem como substrato específico o ácido araquidônico.

As PLA_2s do grupo I são encontradas no veneno das serpentes da família Elapidae e Hydrophidae, e também as isoladas do pâncreas de mamíferos. Possuem uma ponte dissulfeto entre os resíduos 11 e 77. As enzimas do grupo II foram primeiramente isoladas de serpentes da família *Viperidae*. Elas não apresentam a ponte dissulfeto entre o resíduo 11 e 77, mas apresentam 6 resíduos adicionais no final da extremidade C-terminal, com uma ponte dissulfeto entre o último aminoácido e o resíduo 50. As enzimas do grupo III são provenientes do veneno de abelha e sua estrutura difere significantemente daquelas encontradas nos grupos I e II (Six e Dennis, 2000).

As enzimas mais bem caracterizadas são as do grupo IB (PLA_2 de pâncreas de mamíferos) e as do grupo IIA (originalmente isoladas do fluido sinovial de pacientes

com artrite reumatóide) (Kramer et al., 1989; Seilhamer et al., 1989). As enzimas do grupo II também têm sido encontradas em diferentes tipos de células, incluindo mitocôndrias de fígado de rato (Kudo et al., 1993).

Tem-se isolado vários outros tipos de PLA_2s que possuem características diferentes e claramente não se encaixam na classificação antiga. Por exemplo, o grupo IV (anteriormente classificado como PLA_2 citosólica humana) possui PLA_2s que têm sido identificadas em uma variedade de células, com massa molecular de 85 kDa e uma aparente preferência pelo íon araquidonato contido nos fosfolipídios das membranas e translocados ao citosol em presença de níveis sub-micromolares de Ca^{2+}.

Duas novas PLA_2s de baixa massa molecular foram clonadas e caracterizadas por Chen et al. (1994). Estas novas proteínas são únicas e contém 12 e 16 resíduos de cisteína em vez do usual 14, sugerindo 6 e 8 pontes dissulfeto, respectivamente. Estas PLA_2s foram originalmente identificadas a partir do cDNA do grupo II e IB, sendo que a posterior análise da seqüência de aminoácidos deduzida destas PLA_2s revelou um alto grau de homologia com as PLA_2s conhecidas e retém muito dos domínios conservados do sitio ativo de ligação ao Ca^{2+}. Estas enzimas têm sido classificadas como grupo V e são liberadas por macrófagos ativados (Balboa et al., 1996).

A maioria das PLA_2s pertencentes ao grupo II possui um resíduo aspartato na posição 49 (Asp49), altamente envolvido com o sítio de ligação ao Ca^{2+}. Porém, em algumas destas enzimas, o resíduo aspartato na posição 49 é substituído por lisina (Lys49), o que impede uma ligação eficiente ao Ca^{2+} (Maraganore et al., 1984). Dessa maneira, o grupo II pode ser subdividido em PLA_2 Asp49 e PLA_2 Lys49, sendo que as últimas são desprovidas de atividade enzimática ou possuem muito baixa atividade, devido à substituição do aspartato por lisina. Além disso, pelo menos duas variantes

com um resíduo de serina ocupando a posição 49 (PLA_2 Ser49) têm sido descritas (Krizaj et al., 1991; Polgár et al., 1996).

Existem evidências significativas de que a atividade catalítica das PLA_2s não é necessária para gerar uma desorganização de membrana (Díaz-Oreiro e Gutiérrez, 1997). Vários trabalhos mostram que as PLA_2s miotóxicas são proteínas básicas e que apresentam um grande conteúdo de resíduos de lisina na região C-terminal. Segundo Gutiérrez e Lomonte (1995) as PLA_2s poderiam se ligar a determinados "sítios" com carga parcial negativa na membrana, que serviriam de ancoragem para as estas proteínas. Unidas assim à membrana celular, poderiam gerar uma desorganização conformacional da membrana, levando a uma alteração na permeabilidade e conseqüente destruição celular. Miotoxinas com estrutura molecular PLA_2 são as principais responsáveis pela necrose do músculo esquelético descrito nas vítimas do envenenamento por serpentes (Mebs e Ownby, 1990). Estudos subseqüentes com a miotoxina II purificada do veneno de *Bothrops asper*, confirmaram que a região 115-129, rica em aminoácidos básicos e hidrofóbicos, estava envolvida não somente com seu efeito miotóxico in vivo, mas também com sua atividade citotóxica (Lomonte et al*.,* 2003).

Neurotoxinas

Os venenos de serpentes são considerados uma mistura de toxinas e enzimas que estão envolvidas na captura e digestão das presas, bem como defesa contra predadores (Hodgson e Wickramaratna, 2002).

Um dos maiores alvos dos venenos de serpentes é o sistema nervoso somático, em particular a junção neuromuscular. A inibição do neurotransmissor neste sítio resulta

na paralisia dos músculos bulbares e oculares, assim como paralisia dos músculos respiratórios (Lalloo, et al., 1996), resultando em morte.

As neurotoxinas de veneno de serpentes que alteram a transmissão no nervo motor terminal tem sido de considerável importância clinica e de pesquisas durante as últimas décadas. Sua estrutura e modo de ação vêm sendo estudados de forma muito extensa. Devido aos maiores avanços nas técnicas da química de proteínas, as neurotoxinas são continuamente isoladas do veneno de diversas espécies de serpentes (Tu, 1996).

Toxinas pré-sinápticas

Um número de neurotoxinas pré-sinápticas têm sido isoladas do veneno de serpentes. Na verdade, elas têm sido identificadas no veneno de três principais famílias de serpentes peçonhentas: Elapidae, Hydrophiidae e Viperidae (subfamília Crotalinae), indicando a importância desta atividade (Harris, 1997). Estas toxinas exibem atividade PLA_2 e evoluíram presumivelmente de um papel principal de digestão da presa, que inclui a imobilização e morte da presa.

As neurotoxinas pré-sinápticas podem ser um polipeptídio de cadeia simples (notexina) ou toxinas que consistem de mais de uma subunidade. Por exemplo, crotoxina, taipoxina e textiloxina consistem de duas, três e cinco subunidades, respectivamente. A maioria dos venenos contém múltiplas isoformas de uma mesma neurotoxina que diferem em algumas regiões de suas seqüências de aminoácidos (Harris, 1997).

Em geral, estas toxinas produzem bloqueio neuromuscular por inibir a liberação de acetilcolina do terminal nervoso. A ação neurotóxica caracterizada pelo bloqueio

neuromuscular (como conseqüência da inibição da liberação da acetilcolina no terminal nervoso) não altera significantemente a sensibilidade da placa motora para a acetilcolina (Hodgson e Wickramaratna, 2002).

A atividade das β-neurotoxinas (toxinas pré-sinápticas) é geralmente caracterizada por um efeito trifásico na liberação da acetilcolina (uma diminuição, seguida por um aumento transitório e em seguida, completo bloqueio) (Su e Chang, 1984; Harris, 1997). As duas fases iniciais parecem ser independentes da atividade fosfolipásica e são particularmente evidentes quando há redução do Ca^{2+} ou aumento do conteúdo de Mg^{2+} na solução do banho (Chang et al., 1977). Entretanto, existem algumas variações entre as β-neurotoxinas em relação a este efeito trifásico e, em particular, a resposta observada em diferentes preparações musculares.

Dois tipos de β-neurotoxinas pré-sinápticas têm sido encontradas no veneno de serpentes: as β-neurotoxinas com atividade catalítica (PLA_2), encontradas nos venenos de serpentes das famílias *Elapidae* e *Viperidae* e as β-neurotoxinas pré-sinápticas facilitatórias, incluindo a dendrotoxina que bloqueia o canal de potássio voltagem-dependente e as fasciculinas. As toxinas do segundo grupo são próprias do veneno de *Dendroaspis.* São polipeptídios com seqüências homólogas aos inibidores de proteinases do tipo Kunitz e com as curare-like pós-sinápticas que são α-neurotoxinas e cardiotoxinas respectivamente (Harvey et al., 1994).

Toxinas pós-sinápticas

São conhecidas como α-neurotoxinas e ligam-se especificamente aos receptores nicotínicos da placa motora, impedindo, portanto a transmissão do impulso nervoso. Estas neurotoxinas se ligam com alta afinidade e especificidade aos sítios de ligação da

acetilcolina nos receptores nicotínicos da membrana do músculo esquelético. O receptor nicotínico é uma proteína de membrana, constituída de cinco subunidades, com a estequiometria de 2α1, 1β1, 1γ e 1δ (Sáez-Briones et al., 1999; Paterson e Nordberg, 2000). O receptor apresenta dois sítios de ligação para a acetilcolina, localizados na interface entre a cadeia α1 e γ e entre a cadeia α1 e δ (Pedersen e Cohen, 1990; Sáez-Briones et al., 1999). Uma vez que estas toxinas interagem com o sítio de ligação de uma maneira cooperativa, por ocupar um ou ambos os sítios, elas inibem a abertura do canal iônico associado com o receptor em resposta ao agonista colinérgico (Paterson e Nordberg, 2000). Desta forma, as α-neurotoxinas bloqueiam a transmissão no músculo esquelético e causam paralisia na presa.

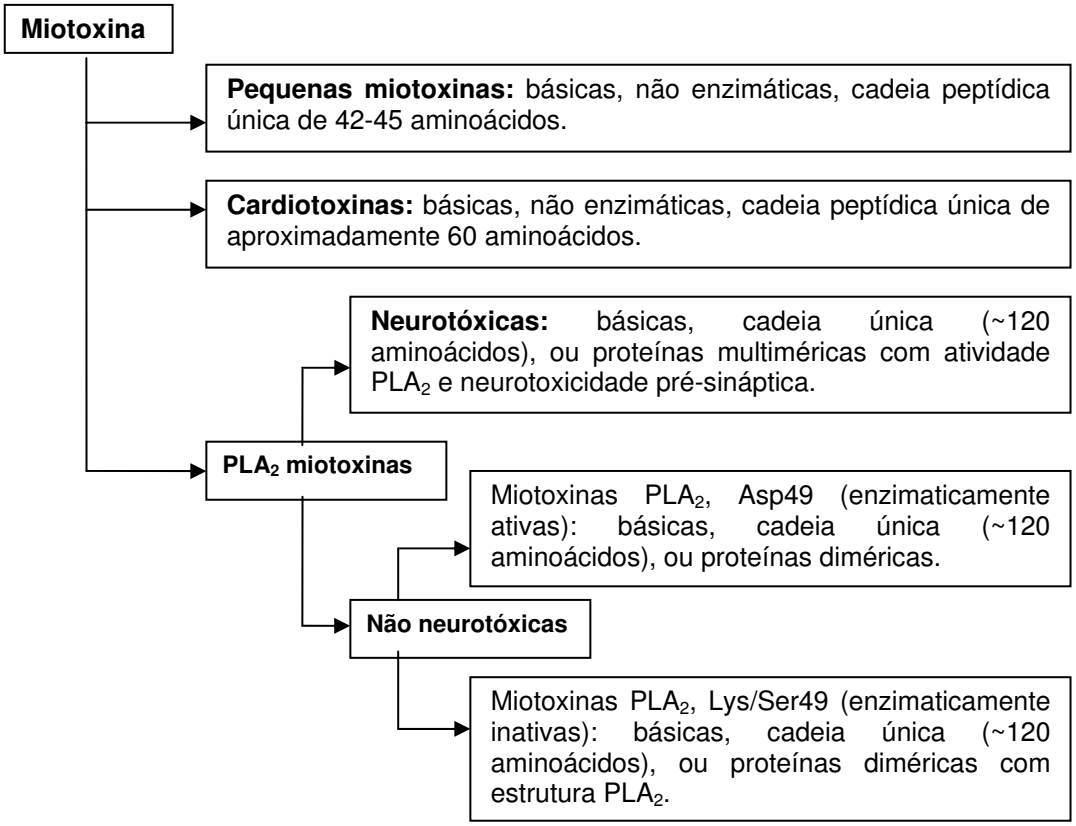
Ao contrário das β-neurotoxinas, as α-neurotoxinas são encontradas somente no veneno de serpentes da família Elapidae e Hydrophiidae (Hodgson e Wickramaratna, 2002). Essas toxinas formam uma extensa família de proteínas homólogas que podem ser divididas em dois subgrupos chamados α-neurotoxinas de cadeia curta e de cadeia longa. As α-neurotoxinas de cadeia curta contém 60-62 resíduos de aminoácidos com quatro pontes dissulfeto, enquanto as α-neurotoxinas de cadeia longa contém 66-74 resíduos de aminoácidos com cinco pontes dissulfeto (Endo e Tamiya, 1991).

Tanto as α-neurotoxina de cadeia curta como as de cadeia longa são estruturalmente homólogas, pois elas têm em comum um número invariável de aminoácidos em sua seqüência que são importantes para a conservação e conformação molecular. A presença e a localização constante das pontes dissulfeto contribuem consideravelmente para a estabilidade da molécula; a atividade letal da toxina é perdida quando ocorre a quebra dessas pontes (Mebs, 1989).

As miotoxinas

Miotoxinas podem ser geralmente definidas como componentes naturais (usualmente proteínas pequenas e peptídeos) de venenos, que induzem danos irreversíveis as fibras do músculo esquelético (mionecrose) após injeção em animais superiores. Algumas miotoxinas atuam localmente, danificando as fibras musculares no local e em áreas próximas da injeção do veneno, entretanto, outras atuam sistemicamente, causando danos musculares em locais distantes a injeção do veneno (Lomonte et al., 2003). Mionecrose é uma importante complicação médica do envenenamento por serpentes. Em alguns casos, a mionecrose local pode levar a seqüelas drásticas, como permanente perda de tecido, inaptidão ou amputação (Milani et al., 1997; Otero et al., 2002). Por outro lado, a miotoxicidade sistêmica (rabdomiólise) pode levar a mioglobinúria e falha renal aguda (Azevedo-Marques et al., 1985), uma freqüente causa de morte do envenenamento ofídico.

Miotoxinas identificadas no veneno de serpentes podem ser classificadas em três grupos principais (Harris e Cullen, 1990) que constituem famílias de proteínas estruturalmente distintas. Estas incluem: (1) as miotoxinas “pequenas” (por exemplo, crotamina de *Crotalus durissus terrificus* e a miotoxina *a* de *Crotalus v. viridis*); (2) as cardiotoxinas e (3) as PLA_2 miotóxicas. As PLA_2 miotóxicas formam o maior grupo, o qual pode ser dividido em neurotóxica e não neurotóxica (Mebs e Ownby, 1990). Entre as não neurotóxicas, uma clara divisão entre “Asp49” e “Lys49” existe, e está detalhada no esquema 1.



Esquema 1. Classificação e características gerais das miotoxinas do veneno de serpentes (Lomonte et al., 2003).

Um quarto grupo de proteínas miotóxicas tem sido considerado (Gutiérrez e Cerdas, 1984), compreendendo uma variedade de componentes do veneno que podem danificar o músculo esquelético por mecanismos indiretos. Como por exemplo, toxinas hemorrágicas que causam danos ao fluxo de sangue local, isquemia e mionecrose secundária, de início mais lento, poderiam ser consideradas como fatores miotóxicos indiretos.

As miotoxinas (PLA_2) neurotóxicas são comumente encontradas no veneno de serpentes do gênero *Elapidae*, onde elas desenvolvem um papel principal no seu efeito

letal geral. Os valores de sua LD_{50} são extremamente baixos, devido a potencia dos efeitos pré-sinápticos na junção neuromuscular (Rosenberg, 1990). Além disso, estas PLA_2s causam impressiva necrose no músculo esquelético a doses muito baixas (1-2 μg) em roedores. Um exemplo bem caracterizado deste grupo de miotoxina é a notexina, do veneno de um elapídeo Australiano, *Notechis s. scutatus* (Harris et al., 1975; Dixon e Harris, 1996). Miotoxinas (PLA_2) neurotóxicas também podem ser encontradas em algumas espécies de viperídeos, como exemplificado pela crotoxina, um componente bem estudado do veneno de *C. d. terrificus* da América do Sul (Hendon e Fraenkel-Conrat, 1971; Gopalakrishnakone et al., 1984; Salvini et al., 2001).

Por outro lado, miotoxinas (PLA_2) não neurotóxicas, são comumente encontradas nos venenos dos viperídeos, onde são notadas como componentes abundantes. Ao contrário das PLA_2 neurotóxicas, estas PLA_2s geralmente exibem altos valores de LD_{50} (Gutiérrez et al., 1986; Homsi-Brandeburgo et al., 1988; Rosenberg, 1990; Angulo et al., 1997), sendo de pequena relevância para o efeito letal completo de seus venenos correspondentes (Lomonte et al., 1985). Suas potências miotóxicas também são menores quando comparada com as PLA_2 neurotóxicas (doses de 25-100 μg são utilizadas em camundongo para mionecrose). Entretanto, devido sua abundancia no veneno de viperídeos/crotalídeos, e a grande quantidade de veneno que é frequentemente injetada nos acidentes, estas miotoxinas são indubitavelmente importantes no desenvolvimento da mionecrose.

OBJETIVOS GERAIS

Este trabalho teve como objetivo geral, aumentar o conhecimento sobre o veneno de *Lachesis m. muta* e sua PLA_2 Asp49 (LmTX-I). Para isso, decidimos:

- Estudar os efeitos do veneno de *L. m. muta* em preparações neuromusculares isolado de ave e mamífero (neurotoxicidade e miotoxicidade) e em cultura de célula *in vitro*.
- Isolar e caracterizar bioquímica e enzimaticamente duas PLA_2 básicas provenientes do veneno de *Lachesis m. muta*
- Caracterizar biologicamente uma PLA_2 (LmTX-I) purificada do veneno de *L. m. muta*, através de um estudo das atividades neurotóxica, miotóxica e citotóxica.

ELSEVIER

Toxicon 46 (2005) 222–229

TOXICON

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Neurotoxic and myotoxic actions from *Lachesis muta muta* (surucucu) whole venom on the mouse and chick nerve–muscle preparations

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Received 3 September 2004; accepted 8 April 2005
Available online 20 June 2005

Abstract

Lachesis genus is one of the less studied among others from Viperidae's genera, mainly due to difficulties in obtaining the venom. Accidents by *Lachesis* snakes cause severe envenoming syndrome, eventually leading victims to shock. This work is part of a comprehensive study aimed at studying the venom and its effects. Herein the neurotoxicity and myotoxicity of *L. muta muta* venom were investigated on mouse phrenic nerve-diaphragm (PNDp) and chick biventer cervicis (BCp) preparations. For both preparations the time required to venom produces 50% neuromuscular blockade was indirectly concentration-dependent, being for PNDp: 117.6 ± 6.5 min (20 μg/ml), 70.1 ± 8.6 min (50 μg/ml) and 43.6 ± 3.8 min (100 μg/ml), and for BCp: 28 ± 1.8 min (50 μg/ml), 30.4 ± 2.3 min (10 μg/ml), 50.4 ± 4.3 min (5 μg/ml) and 75.2 ± 0.7 min (2 μg/ml), (n=5/dose). In BCp, a venom dose of 50 mg/ml significantly reduced contractures elicited by exogenous acetylcholine (55 μM) and KCl (20 mM), as well as increased the release of creatine kinase (442.7 ± 39.8 IU/l in controls vs 4322.6 ± 395.2 IU/l, after 120 min of venom incubation ($P < 0.05$). Quantification of myonecrosis in BCp indicated the doses 50 and 10 μg/ml as significantly myotoxic affecting 59.7 ± 6.2%, and 20.8 ± 1.2% of fibers, respectively, whereas 5 and 2 μg/ml that affected 13.5 ± 0.8% and 5.4 ± 0.6% of fibers, were considered weakly- and non-myotoxic, respectively. We concluded that there are neurotoxins present in the venom, the concentration of which governs its pre- (if low) or postsynaptic (if high) activity. Since myotoxicity in the avian preparation is negligible at lower venom doses, but not neurotoxicity, we suggest that this effect may contribute minimum to the venom neurotoxic effect. The BCp is more sensible than PNDp to *Lachesis m. muta* venom.

Keywords: Biventer cervicis preparation; *Lachesis muta muta* venom; Mouse phrenic nerve-diaphragm; Myotoxicity; Neuromuscular blockade; Neurotoxicity

1. Introduction

In Brazil, the majority of venomous snakes belong to families Viperidae (Crotalinae) and Elapidae. The Viperidae is represented by three main genera: *Crotalus* (rattlesnakes), *Bothrops* (lance-heads) and *Lachesis* (bushmaster, in Brazil

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0041-0101/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2005.04.011

popularly known as surucucu). Envenoming by rattlesnakes frequently causes acute respiratory failure related to neuromuscular paralysis (Vital Brazil, 1972a; Pellegrini Filho and Vital Brazil, 1976), whereas envenoming by Bothrops species causes mainly local effects, represented by edema, pain, hemorrhage and myonecrosis. Although respiratory distress is rare in Bothropic envenoming, it has been already reported (Ribeiro et al., 1988), raising the possibility that *Bothrops* venoms may also exert a neurotoxic action.

The phylogeography of the bushmaster snakes (genus *Lachesis*) has recently been updated using mtDNA sequencing (Zamudio and Green, 1997). Three species and two subspecies have been classified: *L. stenophrys* (Atlantic lowlands of Costa Rica and Panama, Pacific lowlands in Central Panama), *L. melanocephala* (Pacific lowlands of southeastern Costa Rica), *L. muta muta* (equatorial forests of Colombia, Venezuela, Trinidad, Guyana, Suriname, French Guyana, Brazil, Ecuador, Peru and Bolivia) and *L. m. rhombeata* (Atlantic forests of east central Brazil) (Rosenthal et al., 2002). A review on 20 case-reports that occurred in Costa Rica, French Guiana, Brazil, Colombia and Venezuela, of bites in humans, reliably attributed to this snake genus, described the local symptoms of pain, swelling, blistering, and mild coagulopathy, and as similar to those caused by snakebites of the genus Bothrops and other Latin American pit vipers genera, however, early nausea, abdominal colic, repeated vomiting, watery diarrhea and profuse sweating were distinctive symptoms (Jorge et al., 1997), not reported in victims of other New World Crotalinae, but reminiscent of envenoming by Old World Viperine species, such as the European adder *Vipera berus* (Reid, 1976) and Palestine viper, *V. palaestine* (Efrati and Reif, 1953). In Venezuela, a patient developed intense sweating, vomiting, watery diarrhea, hypersalivation, hypotension, bradycardia and respiratory distress 45 min after being bitten by a juvenile bushmaster (Torres et al., 1995). This patient also showed divergent strabismus, dysarthria and dysphagia, symptoms characteristically neurotoxic. These symptoms plus hypotension, bradycardia and shock, which were provoked by Lachesis bites, constituted a type of envenoming syndrome not seen in victims of other American crotaline snakes (Jorge et al., 1997), are possibly caused by autacoids or endogenous substances originated from autonomic nervous system.

Lachesis species live in tropical rain forests of hard access, making difficult its capture and/or maintenance in captivity, what explains literature only dealing with clinical reports and the lack of studies of their venom effects in experimental models.

This study is part of a broader investigation in course of Brazilian bushmaster venom and its effects. Our preliminary focus aims to examine toxicity of *Lachesis muta muta* (surucucu) whole venom effects on neuromuscular system, to further isolate and characterize biochemical and functionally the components of the venom, which may have clinical interest. This study will serve as basis for future studies involving strategies for neutralize the venom and their toxins effects. In the present study, we used mammalian and avian nerve-muscle preparations to examine and characterize the neurotoxicity and myotoxicity of L. m. muta venom. The avian preparation will be used to discriminate myotoxic from neurotoxic venom activities.

2. Materials and methods

2.1. Venom

Desiccated *Lachesis muta muta* venom was purchased from a private serpentarium CETA- Centro de Toxinas Animais (Morungaba, SP, Brazil).

2.2. Animals

Male HY-LINE W36 chicks (4–8 days old) were supplied by Granja Ito S/A (Campinas, SP, Brazil) and had free access to food and water. All procedures were done in accordance with the general guidelines proposed by the Brazilian Council for Animal Experimentation (COBEA), protocol 677–1. 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 ºC on a 12 h light/dark cycle and had free access to food and water.

2.3. Mouse phrenic nerve-diaphragm muscle preparation (PNDp)

The phrenic nerve-diaphragm preparations (Bülbring, 1946) were obtained from mice anesthetized with chloral hydrate (300 mg/kg, i.p.) and sacrificed by exsanguination. 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 ºC) 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 X 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 PNDps were allowed to stabilize for at least 20 min before addition of *L. m. muta* venom (100, 50, or 20 μg/ml).

2.4. Chick biventer cervicis muscle preparation (BCp)

Male chicks (4–8 days old) were killed with halothane inhalation and the biventer cervicis muscles were removed

(Ginsborg and Warriner, 1960) and mounted under a tension of 1.0 g in a 5 ml organ bath containing warmed (37 ºC), 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 BCps were allowed to stabilize for at least 20 min before addition of ACh or KCl, and further venom incubation (concentrations of 50, 10, 5 or 2 μg/ml). Control experiments were done using Krebs solution. Contractures to exogenously applied acetylcholine (ACh, 55 μM for 60 s) and KCl (20 mM for 180 s) were obtained in the absence of field stimulation prior to addition of venom and in the end of the experiment.

2.5. Creatine kinase release

Electrically stimulated BCps were incubated with venom as described above. At 0, 15, 30, 60, 90 and 120 min after venom addition, samples of the bath solution (30 μl) were collected and stored at 4 ºC. Creatine kinase (CK) activity was measured using commercial kit (Bioclin, Labcenter, Campinas, Brazil). The samples were replaced with an equal volume of fresh solution. Enzyme activity was expressed as international units/liter (IU/l), and will be used as a quantitative biomarker to measure (biochemically) fiber damage, and hence venom myotoxic potency.

2.6. Morphological and morphometrical analysis

After complete neuromuscular blockade of BCp incubated with 2, 5, 10 or 50 μg/ml of *L. m. muta* venom, the preparations (n=5) were fixed in Bouin's solution for 24–48 h (control preparations were incubated in Krebs solution for 120 min, n=5), followed by washing with ammonium hydroxide. The muscles were then dehydrated in ethanol series and embedded in historesin. After resin polymerization (60 ºC), the blocks of muscle were sectioned with a microtome (Leica model RM 2145, Leica Microsystems Ltd, Heerbrugg, Switzerland). Sections 2 μm thick were stained with toluidine blue and examined by light microscopy using an Olympus microscope (Olympus Optical Co. Ltd, Tokyo, Japan) prior to photographing. The extent of muscle damage was assessed qualitatively and quantitatively by counting 70 fibers (normal and damaged) in four non-overlapping areas in a total of 280 fibers per preparation. BCps incubated with 2, 5 or 10 μg/ml of venom (n=5) had a total of 1400 fibers counted, whereas BCps incubated with 50 μg/ml of venom (n=4) had a total of 1120 fibers counted. A same procedure was done for the control preparations (n=4; 1120 fibers counted). The percentage of damaged fibers was calculated to assess morphologically the venom myotoxic potency, so permitting correlation with biochemical CK release determination.

2.7. PLA_2 Activity

PLA_2 activity was measured using the assay described by Holzer and Mackessy (1996), modified for 96-well plates according to Bonfim et al., 2001. The standard assay mixture contained 200 μl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 μl of substrate, 20 μl of water, and 20 μl of whole venom in a final volume of 260 μl. After addition of venom (20 μg), the mixture was incubated for up to 40 min at 37 ºC, with the absorbance being read at10 min intervals. Enzyme activity assessment in the venom of surucucu, expressed as the initial velocity of the reaction (V_0), was calculated based on the increase in absorbance after 20 min.

2.8. Statistical analysis

Each experimental protocol (twitch tension records for PNDp or BCp, CK bath content and muscle fibers counting) was repeated at least five times, and the results reported as the mean ± SEM. ANOVA followed by Tukey-Kramer multiple test was used for statistical comparison of the data. A value of $P<0.05$ was considered to indicate significance.

3. Results

3.1. Neuromuscular effect of *Lachesis muta muta* venom on mouse phrenic nerve-diaphragm muscle preparation (PNDp)

The venom (20, 50 or 100 μg/ml) caused a progressive dose-dependent neuromuscular blockade in PNDp indirectly stimulated (Fig. 1). With the lower dose the blockade was only achieved after 100 min incubation, whereas with the higher dose a total and irreversible blockade of the twitch tension took place and was sustained even after several washing ($P<0.05$). The time required for 50% paralysis was 43.6±3.8 min (100 μg/ml), 70.1±8.6 min (50 μg/ml) and 117.6±6.5 min (20 μg/ml) (n=5/dose). In a concentration of 10 μg/ml, 50% blockade was not achieved even after 120 min incubation (data not shown). Control preparations maintained the twitch tension records stable at 98% throughout the 120 min of incubation with Tyrode solution (n=5).

3.2. Neuromuscular effect of Lachesis muta muta venom on chick biventer cervicis muscle preparation (BCp)

All the tested venom concentrations (2, 5, 10, or 50 μg/ml) caused an irreversible dose-dependent blockade

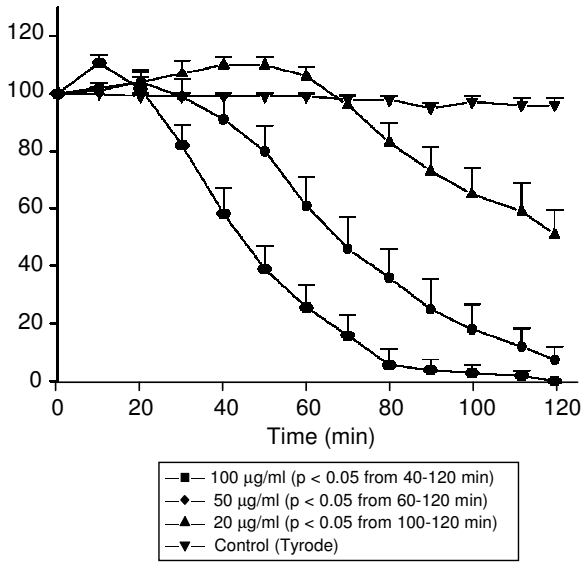

Fig. 1. Neuromuscular blockade induced by the whole venom from *Lachesis muta muta* on the mouse phrenic nerve-diaphragm preparation. Each point represents the average from five experiments ±SEM. $P< 0.05$ compared to control.

of the neuromuscular transmission ($P<0.05$) (Fig. 2). Concentration of 10 µg/ml represented the maximum dose needed for a neuromuscular blockade, as its twitch tension record profile coincided with the evoked by 50 µg/ml. The time required for achieving 50% twitch tension blockade, through an indirect stimulation was 28±1.8 min (50 µg/ml), 30.4±2.3 min (10 µg/ml), 50.4±4.3 min (5 µg/ml) and 75.2±0.7 min (2 µg/ml), (n=5/dose). The twitch tension records of control preparations remained stable at 94% all along the 120 min incubation in Krebs solution without venom (n=5).

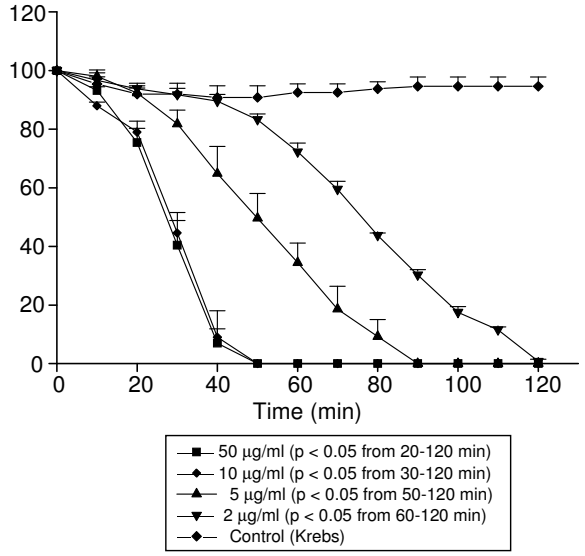

Fig. 2. Neuromuscular blockade induced by the whole venom from Lachesis muta muta on the chick biventer cervicis muscle preparation. Each point represents the average from five experiments±SEM. $P< 0.05$ compared to control.

3.3. ACh- and KCl-induced contractures in BCp before and after venom addition

At the highest concentration used (50 µg/ml), the venom significantly altered the ACh- (55 µM) and KCl- (20 mM) induced contractures when compared to the control values (Fig. 3). Complete blockade at lower concentrations (10, 5 or 2 µg/ml) was not accompanied by inhibition of the response to ACh. However, the KCl-induced contracture at 10 µg/ml was significantly inhibited by the whole venom. At the lowest concentrations (5 or 2 µg/ml) the venom did not significantly alter the ACh- and KCl-induced contractures when compared to control values (Fig. 3). In the control preparations, the contracture to ACh and KCl was kept stable, after a 120 min indirect stimulation (data not shown).

3.4. Myotoxic activity of *Lachesis muta muta* venom (CK release *in vitro*)

As shown in Fig. 4, the whole venom from *L. m. muta* caused a dose and time-dependent increase in the rate of CK release from isolated BCp, except with 2 and 5 µg/ml doses that showed no difference among them. However, a significant increase in CK release was caused by 10 µg/ml of venom either in short (15 min) or longer (120 min) period of incubation. The medium of CK release ranged from 1348 IU/l (2 and 5 µg/ml) to 4322 IU/l (50 µg/ml).

3.5. Morphological changes

Chick BCp incubated with Krebs solution alone (controls) showed normal muscle morphology with regular muscle fiber fascicles and uniform intensity of cells staining.

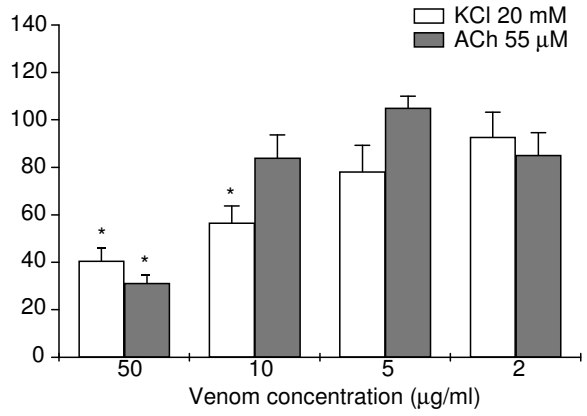

Fig. 3. Effect of the whole venom from Lachesis muta muta on the response of the chick biventer cervicis preparation to the acetylcholine (ACh) and potassium (KCl). Each point represents the average from five experiments±SEM. *P<0.05 compared to control.

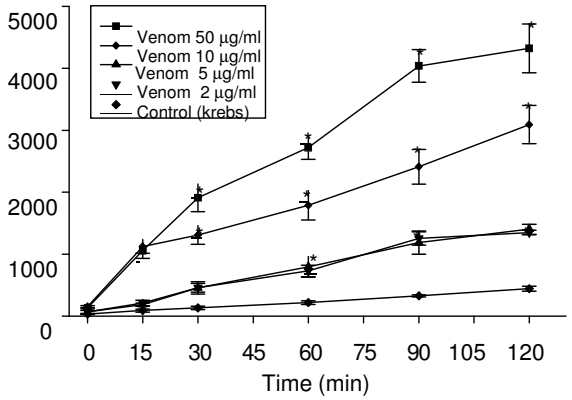

Fig. 4. Creatine kinase (CK) release from chick BCp following incubation which *L. m. muta* venom (2, 5, 10 or 50 µg/ml). The venom was added to the bath at time zero and the CK release expressed as means±SEM of five experiments. **P*<0.05 compared to control.

Cross-sections of fibers showed the characteristic punctuations that result from cross sectioning of clusters of myofibrils (Fig. 5A). Muscles incubated with 10 or 50 µg/ml of *L. m. muta* venom showed disorganization of the fascicles and several darker compacted cells, where punctuations derived from cross-sectioned myofibrils clusters were missed. Abnormal looking cells, vacuolated and heterogeneously sized were indirect evidences of membrane lesions (Fig. 5B and C). A quantitative difference (and at some extent, also a qualitative one) was observed in relation to 10 and 50 µg/ml myotoxic potency in BCp. A direct correlation between venom dose and percentage of damaged fibers is confirmed by the quantitative analysis as follow: 50 (59.7±6.2%), 10 (20.8±1.2%) and 5 µg/ml (13.5±0.8%), respectively. In the dose of 2 µg/ml (5.4±0.6%), the percentage of fibers affected did not differ significantly from control (1.07±0.4%).

4. Discussion

Neurotoxins from snake venoms that cause neuromuscular paralysis act either pre-junctionally by blocking acetylcholine (ACh) release or post-junctionally by blocking nicotinic receptors. These different mechanisms of action cannot be easily differentiated using the mouse phrenic nerve-diaphragm preparation (PNDp), but they can be differentially demonstrated using the chick biventer cervicis preparation (BCp) (Harvey et al., 1994).

Snake myotoxins, usually small proteins and peptides, can be generally defined as natural components of venom gland secretions that induce damage at the site of the bite, the extension of which can irreversibly cause permanent muscle tissue loss, disability, and amputation in the victims (Roselfeld, 1971; Kerrigan, 1991; Nishioka and Silveira, 1992). Therefore, a growing interest on venom components responsible for myonecrosis and their mode of action has

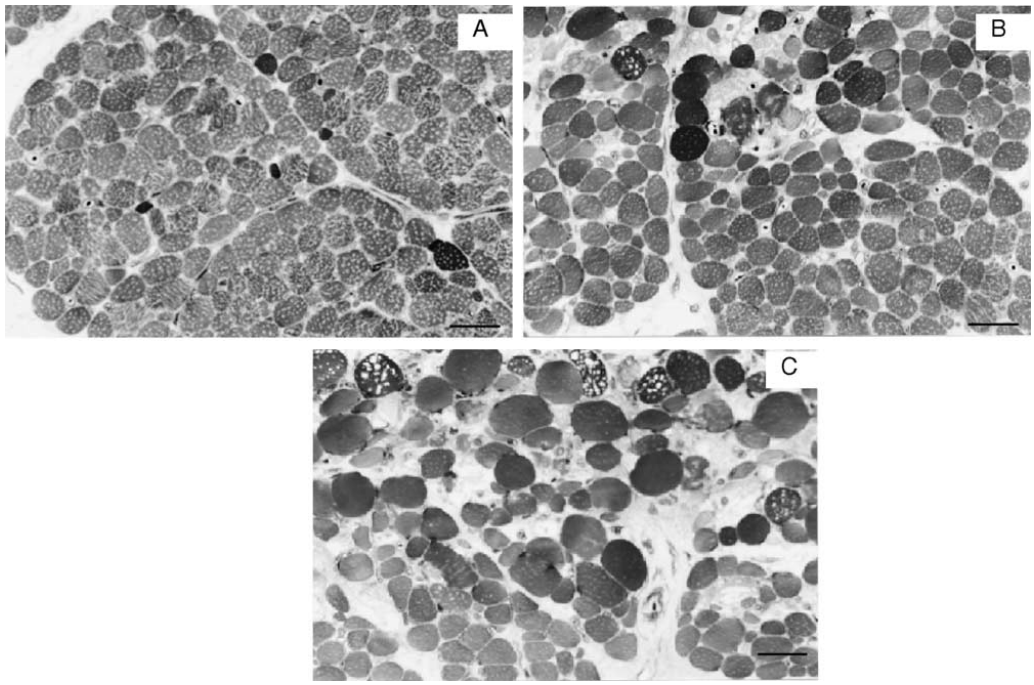

Fig. 5. Light micrographs of chick BCp (cross-sections). (A) BCp incubated with Krebs solution alone after 120 min incubation (control). Note the regular fiber fascicles and punctuations of myofibrils cut transversally in individual fibers. Rare cells are dark cells. (B) BCp incubated with *L. m. muta* venom (10 µg/ml), and (C) (50 µg/ml). Note that the higher the venom dose, higher the number and polymorphism of altered fibers. Likewise the loss of visible bundles of cross-sectioned myofibrils is patent. Toluidine blue. Bar=25 µm for both panels.

happened during the last few decades (Gutiérrez and Ownby, 2003).

Herein we report the neurotoxic and myotoxic activities of the venom of one of the most important subspecies from genus *Lachesis*, *L. m. muta*, and that these activities are dose-dependent both in the PNDp and BCp. We also show that the avian preparation is more susceptible to the venom than the mammalian one, at the conditions studied. For instance, while it is necessary 100 μg/ml of venom for PNDp achieve neuromuscular blockade in approximately 100 min, for the BCp a dose of 10 μg/ml leads to blockade at about 50 min. Likewise, while it takes 120±4.6 min for a total blockade of PNDp with a dose of 50 μg/ml, only 2 μg/ml of venom is necessary for BCp blockade in this very same period of time. In agreement with our results, Serafim et al. (2002) showed that *Micrurus dumerilli carinicauda* venom (Elapidae) is also more active in BCp (5 μg/ml venom concentration caused neuromuscular blockade in 90 min) than in PNDp (5 μg/ml concentration caused blockade in 291.8±7.3 min). The mechanism that could explain such differences was not studied yet.

Another difference, observed only in mammalian preparation was that *L. m. muta* venom induced a discrete transient increase of indirectly evoked twitch tension before development of a progressive neuromuscular blockade. This facilitation was not seen in BCp. β-neurotoxins activity typically is characterized by a triphasic effect on acetylcholine release (i.e. there is a decrease, followed by a transient increase and then a complete blockade) (Hodgson and Wickramaratna, 2002). The authors also report that some variability exists in the response of different muscle preparations regarding this triphasic activity of β-neurotoxins. For example, although paradoxin from the venom of the inland taipan (*Oxyuranus microlepidotus*) produces marked facilitation of twitch tension in the mouse phrenic nerve-diaphragm muscle preparation, the initial inhibitory phase appears to be absent (Hodgson and Rowan, 1997), in agreement with our results for PNDp.

Using an avian neuromuscular preparation we have shown that median (5 μg/ml) and low (2 μg/ml) venom concentrations of *L. m. muta* were able to produce potent neurotoxic activity, leading to 50% twitch tension blockade after 50.4±4.3 and 75.2±0.7 min, respectively, followed by a total and irreversible blockade. PNDp probably would no be affected by these concentrations, since with 20 μg/ml the blockade was achieved only at 117.6±6.5 min. Interestingly, these same concentrations produce only very mild or none myonecrosis-inducing effects in BCp. The results indicate that the neurotoxic effects observed with 5 and 2 μg/ml in BCp were independent from any myotoxic venom effect, and that low venom concentrations have proven to be useful to detect neurotoxic effects, since can eliminate, or reduce to minimal, myotoxic undesirable effects that could mask neurotoxicity.

Neurotoxic effect uses to be typical of low doses of venom from crotalids. To induce 50% twitch tension blockade in BCp, 10 μg/ml of *C. d. terrificus* venom spent 16.3±0.7 min (Rodrigues-Simioni et al., 2004) and 5 μg/ml of *C. d. cascavella* venom spent 24.8±1.6 min (Beghini et al., 2004). In comparison, using corresponding doses, *L. m. muta* venom took double the time to achieve 50% blockade as that of *C. d. terrificus* (10 μg/ml), and *C. d. cascavella* (5 μg/ml) venom in the same preparation. Comparatively, these finding suggest that *L. m. muta* venom has lesser neurotoxic potency than crotalic venoms, but at same time resemble Crotalus venom because at very low concentration the neurotoxicity is displayed.

Our findings showed that complete blockade with 5 or 2 μg/ml was not accompanied by inhibition of the responses to KCl and ACh. A presynaptically active neurotoxin would abolish nerve-evoked twitches without affecting response to cholinoreceptor agonists (Lewis and Gutmann, 2004). These observations suggest that at lower concentrations the *L. m. muta* venom had no inhibitory effect on postsynaptic acetylcholine receptors. Since only ~5–10% of fibers were affected with venom doses ranging from 2 to 5 μg/ml, we suggest, as mentioned above, that with these doses myotoxicity does not contribute for neurotoxicity in general and for presynaptic effect in particular. CK values support this hypothesis. However, at the highest concentration (50 μg/ml) a pronounced muscle contracture, concomitant with inhibition of the response to ACh and KCl, was observed, pointing though to a postsynaptic effect. The fact the whole venom from *L. m. muta* did not significantly affect ($P<0.05$) the response to ACh and KCl, unless a higher dose is used, suggests the venom primordial presynaptic nature. In this sense, *L. m. muta* venom behaves as *C. d. terrificus* venom, which at 10 μg/ml concentration does not inhibit the response to ACh, and likely points to a preponderant presynaptic action (Rodrigues-Simioni et al., 2004).

Regarding *L. m. muta* venom myotoxicity, it was well expressed when high (50 μg/ml) and median (10 μg/ml) concentrations of venom were used. Both doses provoked significant CK release and inhibition of KCl-induced contractures, besides to cause damage in 59.7±6.2% and 20.8±1.2% fibers, respectively. It is reasonable to think that higher the venom concentration higher will be the contribution of the venom myotoxins to cooperate with neurotoxins to abolish twitch tension muscle responses, since lesser fibers will remain intact. For *L. m. muta* venom at 50 and 10 μg/ml approximately 40 and 80% of fibers remains intact, respectively. Recently, Borja-Oliveira et al. (2003) reported similar effects for the *B. neuwiedii pauloensis* venom (10, 20 or 50 μg/ml), and Beghini et al. (2004) verified that *C. d. cascavella* venom produced an extensive muscular damage only when 25 μg/ml was used.

As for a parallelism between the amount of CK released and the extent of muscle fibers damaged, there was correspondence in venom concentrations ranging from 5 to 50 μg/ml, but not with 2 μg/ml. At this lowest venom concentration used the number of muscle fibers affected was

5.4±0.5% (~95% of fibers remained unaffected) which was not significantly different from the controls, but there was significant difference between controls and 2 μg/ml-treated BCp, in relation to CK levels. A clear explanation for the lack of a good correlation between the biochemical and morphological parameters used to estimate the myonecrotic effects has been already reported elsewhere (Suarez-Kurtz, 1982; Mebs et al., 1983; Oshima-Franco et al., 1999). Necrosis following snakebite is a very complex phenomenon, because involves the direct action of muscle damaging toxins and/or can result indirectly from secondary tissue reactions. In this sense, to correlate intensity of damage seen by light microscopy with CK levels may not be an easy task. According Mebs et al. (1983) this correlation is easier to be accomplished with pure toxins.

We suggest that some characteristics of *Lachesis m. muta* venom resemble bothropic venoms, as the myotoxicity, and others resemble Crotalic venoms, as neurotoxicity. Literature on *Lachesis* venom using this type of methodology is deficient, therefore, not permitting comparison intra-genus.

Muscle damage is one of the most common local effects produced by venoms from Viperidae species. Herein, we saw that *L. m. muta* venom has component(s) potentially myotoxic. Morphological and biochemical indirect evidences suggest that the venom disrupt sarcolemma integrity. These effects can be attributable to proteolytic venom components that may or may not be associated with phospholipase A_2 (PLA_2) activity. PLA_2 activity in *L. m. muta* venom has already been reported by Fortes-Dias et al. (1999), and was confirmed in our venom pool using the synthetic substrate 4-nitro-3-(octanoyloxy) benzoic acid (Holzer and Mackessy, 1996). Many bothropic venoms and toxins cause myonecrosis (Lomonte et al., 2003) attributable to the presence of PLA_2 that could cause lysis of the sarcolemma. PLA_2 may be also neurotoxic, like the F15 (PLA_2) isoform from *C. d. terrificus* venom that showed significant blocking effect in avian nerve-muscle preparation (Toyama et al., 2003). Whether the PLA_2 activity of *L. m. muta* venom is responsible for myotoxic or neurotoxic or both actions will be a matter of investigation of our research group.

Lachesis m. muta venom caused a time and dose-dependent increase in CK release in chick BCp, being more potent than venoms from *B. leucurus* (10 μg/ml) and *B. jararaca* (20 μg/ml), respectively (Prianti et al., 2003). At a same venom concentration, *L. m. muta* increased CK level in 3092.3±309 IU/l against the 800 IU/l from *B. leucurus* (120 min), showing that besides being neurotoxic it also presents a not negligible myotoxic activity. The CK content in the incubation bath provided an additional parameter for confirming cell membrane impairment caused by surucucu venom, as proved by histological examination of heteromorphic damaged fibers, mainly seen at 50 μg/ml. Morphologic alterations, like the observed in this work have been described after envenoming by myotoxins-containing venoms (see Gutiérrez and Lomonte, 2003, for revision), or experimentally using PLA_2 isolated from snake venoms (see Gutiérrez and Ownby, 2003, for revision).

In conclusion, our in vitro experimental models have proven be valuable tools to examine actions of *L. m. muta* whole venom. It became clear that the venom has pharmacologically active components that act on neuromuscular junction and muscle fibers, whose intensity of action will depend on the venom concentration and on the type of nerve–muscle preparation (mouse or chick). Low venom concentrations in BCp permitted perceive that venom has presynaptic neurotoxins, whereas at higher concentrations (50 μg/ml), the postsynaptic ones were expressed (inhibition to ACh and KCl responses). The neurotoxic action overcomes the myotoxic action, since at lower doses neurotoxicity is evidenced while myotoxicty is not, or is subtle. *Lachesis m. muta* venom action shares some effects with *Crotalus* venoms and others with *Bothrops* venom.

Acknowledgements

D. C. S. D. is a recipient from a Doctoral fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior). This work was also supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). The authors thank Mr Gildo Bernardo Leite, Mr Paulo Baldasso and Mrs Cleuza Oliveira Franco for technical support.

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Biochimica et Biophysica Acta 1726 (2005) 75 – 86

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Biochemical and enzymatic characterization of two basic Asp_{49} phospholipase A_2 isoforms from *Lachesis muta muta* (Surucucu) venom

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Received 6 April 2005; received in revised form 20 May 2005; accepted 20 May 2005
Available online 17 June 2005

Abstract

Two basic phospholipase A_2 (PLA_2) isoforms were isolated from *Lachesis muta muta* snake venom and partially characterized. The venom was fractionated by molecular exclusion chromatography in ammonium bicarbonate buffer followed by reverse-phase HPLC on a C-18 μ-Bondapack column and RP-HPLC on a C-8 column. From liquid chromatography-electrospray ionization/mass spectrometry, the molecular mass of the two isoforms LmTX-I and LmTX-II was respectively measured as 14,245.4 and 14,186.2 Da. The pI was respectively estimated to be 8.7 and 8.6 for LmTX-I and LmTX-II, as determined by two-dimensional electrophoresis. The two proteins were sequenced and differentiated from each other by a single amino acid substitution, Arg_{65} (LmTX-I) $\longrightarrow$ Pro_{65} (LmTX-II). The amino acid sequence showed a high degree of homology between PLA_2 isoforms from *Lachesis muta muta* and other PLA_2 snake venoms. LmTX-I and LmTX-II had PLA_2 activity in the presence of a synthetic substrate and showed a minimum sigmoidal behaviour; with maximal activity at pH 8.0 and 35–45 °C. Full PLA_2 activity required Ca^{2+} and was respectively inhibited by Cu^{2+} and Zn^{2+} in the presence and absence of Ca^{2+}. Crotapotin from *Crotalus durissus cascavella* rattlesnake venom significantly inhibited ($P<0.05$) the enzymatic activity of LmTX-I, suggesting that the binding site for crotapotin in this PLA_2 was similar to another in the basic PLA_2 of the crotoxin complex from *C. durissus cascavella* venom.

Keywords: Characterization; Enzyme; *Lachesis muta muta*; Phospholipase A_2; Snake venom

1. Introduction

Viperidae snakes of the subfamily Crotalinae are represented in South America by three genera: *Crotalus*, *Bothrops* and *Lachesis*. *Lachesis muta muta* is the largest crotalinae snake species in the world (attaining 3.0–3.5 m). Their habitat is the Amazon tropical forest in Brazil [1]. These snakes are popularly named Surucucu (from the native Brazilian indigenous Tupy-Guarani Suú-u-u, a snake that gives many bites, many attacks), Surucucu Pico-de-Jaca, and Surucutinga [2].

The main pathological effects of the *L. muta* venoms include proteolytic, haemolytic, haemorrhagic, myotoxic, coagulant, defibrinant and fibrinolytic. When there is an accident with a L. muta and a human, the main consequences include local pain, edema, hemorrhage, necrosis, and systemic disorders such as nausea, vomiting, diarrhoea, hypotension and bradicardia, coagulation disturbs and renal malfunction [3]. *L. muta muta* venom significantly has higher levels of procoagulant, proteolytic and phospholipase A_2 activities in comparison with several *Bothrops* snake venoms [4].

Phospholipases A_2 (PLA_2, E.C. 3.1.14) are calcium-dependent enzymes that hydrolyze the two-ester bonds of 1,2-diacyl-3sn-phosphoglycerides. They display a wide variety of pharmacological activities such as presynaptic/

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doi:10.1016/j.bbagen.2005.05.022

postsynaptic neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, antiplatelet, convulsant, hypotensive, haemolytic, hemorrhagic and edema-inducing effects [5]. Secretory phospholipases A_2 ($sPLA_2$) are classified into three groups according to their primary structure. Group I PLA_2s is composed of both mammalian pancreas and snake venoms belonging to the Elapidae and Hydrophidae families, whereas group II PLA_2s is formed by venoms from Crotalidae and Viperidae families. Group III PLA_2s has mainly been isolated from bee (*Apis mellifera*) and lizard venoms [6]

Phospholipases A_2 are one of the most extensively studied and characterized proteins. However, the efforts on the isolation and characterization of venom PLA_2 enzymes have so far been directed toward the venoms of snakes from the genus *Crotalus* and *Bothrops* [7]. Minimum attention has been paid to the isolation and characterization of PLA_2 from the *L. muta* venom; only two acid PLA_2 isoforms presented in *L. muta* venom were purified [8,9].

The paper is focused on the isolation and enzymatic and biochemical characterization of two basic D49 PLA_2 isoforms termed LmTX-I and LmTX-II from the venom of *L. muta muta*. The sequence of LmTX-I and LmTX-II has respectively been determined in 86% and 84%, representing the most complete sequence data from *L. muta* venom PLA_2.

2. Materials and methods

2.1. Venom and reagents

The venom and the solvents (HPLC grade), 4-nitro-3 (octanoyloxy) benzoic acid, sequence grade bovine pancreatic trypsin and other reagents were from Sigma Chemical Co. (St. Louis, MO, USA). The water, buffers and solvents used in the purification protocols were filtered through 0.22 μm filters (Millipore) and degassed by sonication. RP-HPLC was made using a μ-Bondapack C18 column (0.78 cm x 30 cm) and a Jones Chromatography Genesis C8 column (0.46 cm x 15 cm).

2.2. Molecular exclusion chromatography on a Superdex 75 column

L. muta muta venom (45 mg) was dissolved in 0.2 M ammonium bicarbonate pH 8.0, and loaded onto a Superdex 75 column (1 cm x 60 cm) previously equilibrated with the same solution. The proteins were eluted at a constant flow rate of 0.3 ml/min and the elution profile was monitored at 280 nm. The fraction containing PLA_2 activity was pooled, lyophilised, and conserved at 20 °C.

2.3. Reverse-phase HPLC (RP-HPLC)

Five milligrams of the PLA_2 fraction was dissolved in 200 μl of 0.1% (v/v) trifluoroacetic acid (solvent A). The resulting solution was clarified by centrifugation and the supernatant was applied to a μ-Bondapack C18 column (0.78 cm x 30 cm; Waters 991- PDA system). Fractions were eluted using a non-linear gradient (0–54%, v/v) of acetonitrile (solvent B) at a constant flow rate of 1.0 ml/min over 40 min and the resulting fractions were manually collected. Fractions containing protein species active against the PLA_2 assay were submitted to a new RP-HPLC purification step performed with a Jones Chromatography Genesis C8 column (0.46 cm x 15 cm), with a linear gradient from 20% to 35% B over 90 min at a constant flow rate of 1.0 ml/min. The elution profile of both analysis was monitored at 220 and 280 nm, and the collected fractions were lyophilised and conserved at 20 °C.

2.4. 2D-PAGE analysis

The lyophilized isoforms (300 μg) were diluted to a final volume of 350 μl with a solution containing 8 M urea, 4% w/v CHAPS, 2% v/v carrier ampholytes, pH 3–10, 70 mM DTT and 0.001% w/v BPB. Except for CHAPS from Sigma, all reagents were obtained from Amersham Biosciences (Uppsala, Sweden). After centrifugation at 14,000 xg for 4 min, the supernatant was used for the first dimension run. Samples were applied to IPG gel strip with a nonlinear separation range of 3–10 (Amersham Biosciences). After a 12 h rehydration, IEF was carried out at 20 °C, for 1 h at 500 V, for an additional hour at 1000 V and then for 12 h at 8000 V in an IPGphor apparatus (Amersham Biosciences), maintaining a limiting current of 50 μA per strip. First dimension strips were subjected to the standard reduction and alkylation steps prior to second dimension electrophoresis. The strip was soaked for 8 min in a buffer containing 100 mM Tris–HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS and 2% w/v DTT, and additionally for 12 min in the same buffer containing 2.5% w/v iodoacetamide substituting the DTT. SDS-PAGE was performed on 12.5% T polyacrilamide gel run on a SE-600 system connected to a Multitemp II refrigerating system (Amersham Biosciences). The IPG gel strip was sealed to the surface of the second dimension gel using 0.5% w/v agarose. Electrophoresis was carried out for 1 h at 60 V when a constant current of 30 mA was applied until the dye front reached the lower end of the gel. Proteins were visualized using Coomassie blue staining.

2.5. PLA_2 activity

PLA_2 activity was measured using the assay described by Cho and Kezdy [10] and Holzer and Mackessy [11], modified for 96-well plates [12]. The standard assay mixture contained 200 μl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 μl of substrate, 20 μl of water, and 20 μl of PLA_2 in a final volume of 260 μl. After adding PLA_2 isoforms (20 μg), the mixture was incubated for up to 40 min at 37 °C, with the reading of absorbance at intervals of 10 min. The enzyme activity, expressed as the

initial velocity of the reaction (V_0), was calculated based on the increase of absorbance after 20 min. The main isoform (LmTX-I) obtained from RP-HPLC on the C8 column was chosen by studying the kinetic parameters. The inhibition of PLA_2 activity by crotapotin from *Crotalus durissus cascavella* was determined by incubating both proteins (LmTx-I and crotapotin) for 30 min at 37 ºC prior to assaying the residual enzyme activity. The pH and optima temperature of the PLA_2 were determined by incubating the enzyme in four buffers of different pH values (4–10) and in Tris–HCl buffer, pH 8.0, at different temperatures, respectively. The effect of substrate concentration (10, 5, 2.5, 1.25, 0.625 and 0.312 mM) on enzyme activity was determined by measuring the increase of absorbance after 20 min of incubation in Tris–HCl buffer, pH 8.0, at 37 ºC. All assays were done in triplicate and the absorbances at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA).

2.6. Reduction and alkylation

Purified lyophilised protein from RP-HPLC was resuspended in 8 M urea containing 10 mM DTT at pH 8.0 and the disulfide bridges were then reduced by incubation at 37 ºC for 2 h. Since the number of cysteine residues in the protein was initially unknown, the optimum concentration of iodoacetamide for alkylating the free thiols was derived empirically, based on results obtained from incubations using various concentrations of iodoacetamide and different amounts of protein, with each mixture being analysed by mass spectrometry [13]. Based on these preliminary experiments, a 30% molar excess of iodoacetamide relative to the total number of thiols was eventually chosen and the mixture was incubated for 1.5 h at 37 ºC in the dark. The reaction was ceased by injecting the mixture onto a RP-HPLC column followed by lyophilization of the collected peak.

2.7. Enzymatic hydrolysis

The purified proteins were hydrolysed with sequencing grade bovine pancreatic trypsin in 0.4% ammonium bicarbonate, pH 8.5, for 4 h at 37 ºC, at an enzyme:substrate ratio of 1:100 (w/w). The reaction was ceased by lyophilization.

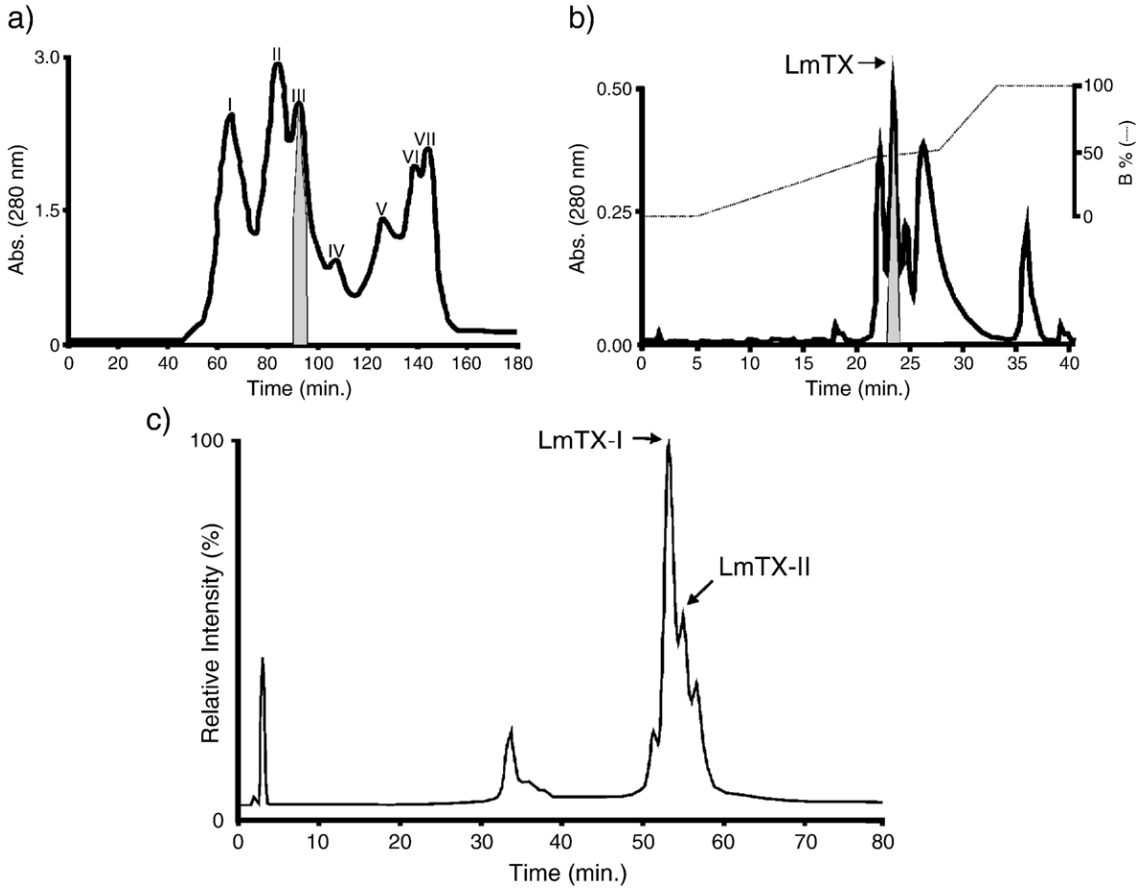

Fig. 1. (a) Elution profile of *L. muta muta* venom by molecular exclusion chromatography on a Superdex 75 column. Fraction III contained PLA_2 activity. (b) Elution profile of FIII following RP-HPLC on a μ-Bondapack C18 column. The peak corresponding to the phospholipase A_2 (LmTX) from *L. muta muta* venom is indicated. (c) RP-HPLC profile of LmTX eluted on a Genesis (Jones chromatography) C8 column. The chromatographic peaks containing PLA_2 active proteins (LmTX-I and II) are indicated.

2.8. Mass spectrometry

All mass spectra were acquired using a quadrupole-time of flight (Q-TOF) hybrid mass spectrometer Q-TOF Ultima from Micromass (Manchester, UK) equipped with a nano Z-spray source operating in a positive ion mode. The ionization conditions of usage included a capillary voltage of 2.3 kV, a cone voltage and RF1 lens of 30 V and 100 V, respectively, and a collision energy of 10 eV. The source temperature was 70 ºC and the cone gas was N_2 at a flow of 80 l/h; nebulizing gas was not used to obtain the sprays. Argon was used for collisional cooling and for fragmentation of ions in the collision cell. External calibration with sodium iodide was made over a mass range from 50 to 3000 m/z. All spectra were acquired with the TOF analyser in "V-mode" (TOF kV = 9.1) and the MCP voltage set at 2150 V.

2.9. Analysis of native and alkylated protein

Lyophilised RP-HPLC fractions of intact native and alkylated protein were dissolved in 10% acetonitrile in 0.1% TFA and was introduced into the mass spectrometer source with a syringe pump at a flow rate of 500 nl/min. Mass spectra were acquired over the mass range of 1000–2800 m/z for the native protein and over the range of 800–2000 m/z for the alkylated protein, both at a scan speed of 1 s/scan. The masses were by the MassLynx-MaxEnt 1 deconvolution algorithm.

2.10. De novo sequencing of tryptic peptides

Alkylated tryptic peptides fractionated by RP-HPLC were lyophilised and resuspended in 20% acetonitrile in 0.1% TFA prior to injection into the mass spectrometer source at a flow rate of 500 nl/min. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 400–2000 m/z, in order to select the ion of interest, subsequently, these ions were fragmented in the collision cell (TOF MS/MS mode). Different collision energies were used, depending on the mass and charge state of the ions. The resulting product-ion spectra were acquired in the TOF analyser and deconvoluted using the MassLynx-MaxEnt 3 algorithm. Singly charged spectra were processed manually using the PepSeq application included in MassLynx.

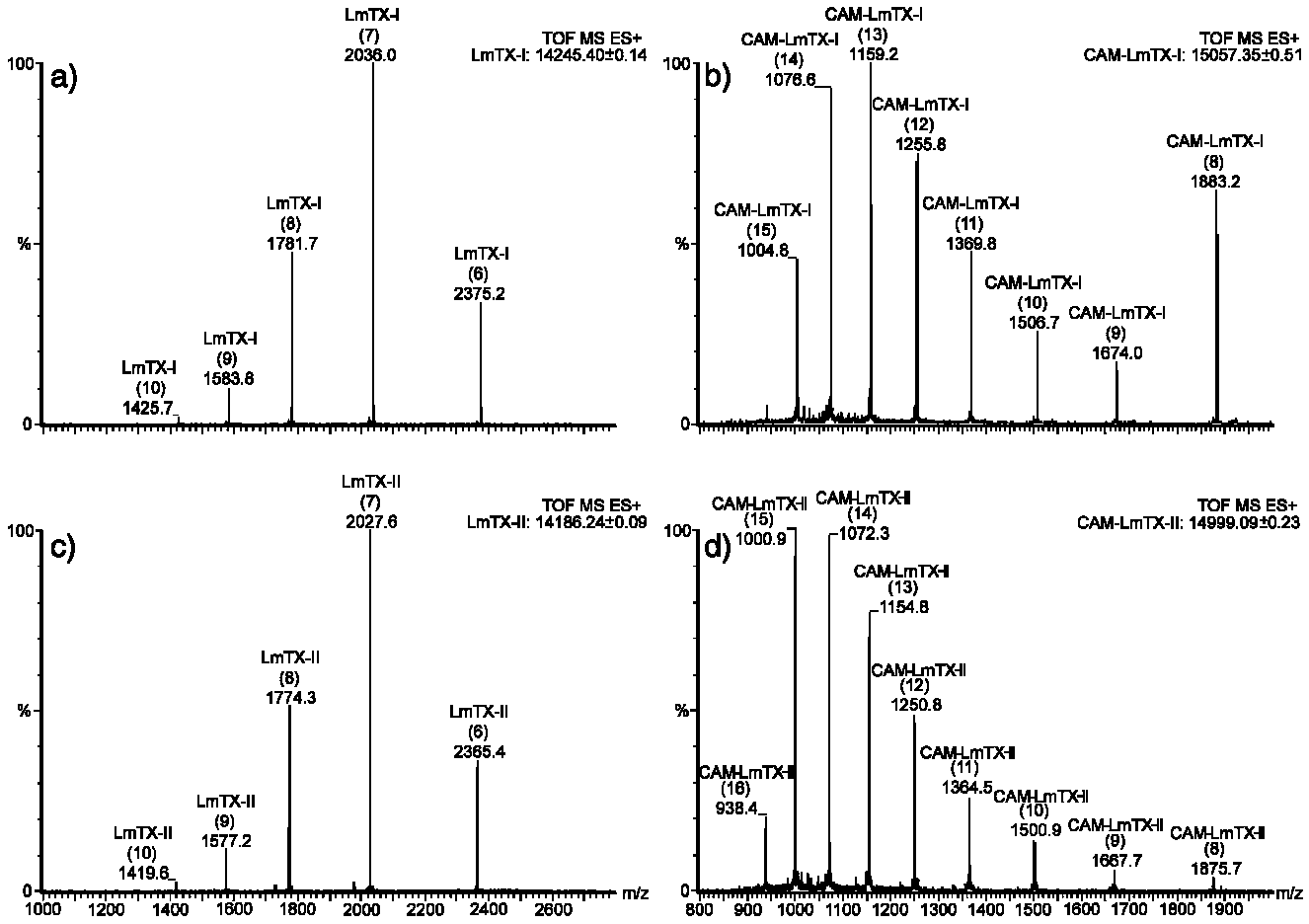

Fig. 2. Raw electrospray positive mass spectrum, showing multicharged ions distributions of native LmTX-I (panel a) and LmTX-II (panel c) and alkylated LmTX-I (panel b) and LmTX-II (panel d).

2.11. Statistical analysis

Each experimental protocol was repeated at least three times, and the results reported as the mean ± S.E. ANOVA followed by the Tukey–Kramer multiple tests were used for statistical comparison of the data. A value of $P < 0.05$ was considered to indicate significance.

3. Results

The fractionation of *L. muta muta* venom by molecular exclusion chromatography on Superdex 75 resulted in seven main fractions (I–VII) (Fig. 1a). Among these seven, only one fraction was present in the PLA_2 activity (Fraction FIII). The proteins contained in this chromatographic peak were further purified using RP-HPLC performed on a C18 column. The elution profile obtained is shown in Fig. 1b. The four eluted peaks were manually collected, lyophilised and screened again for PLA_2 activity. Only the fraction, labelled in Fig. 1b as LmTX, had PLA_2 activity (Fig. 1b).

Electrospray mass spectrometry analysis performed on LmTX peak showed the presence of five different protein species; therefore, a further purification step was performed in order to ascertain these proteins, whose component presented PLA_2 activity. Fig. 1c shows the result of the repurification of active LmTX fraction by RP-HPLC using a C8 column; the chromatogram shows the presence of five main peaks, among these, two PLA_2 active fractions were identified and denominated LmTX-I and LmTX-II.

Both LmTX-I and LmTX-II chromatographic peaks contained a single protein with a molecular mass of 14,245.5 Da (Fig. 2a) and 14,186.2 Da (Fig. 2c), respectively. In order to determine the presence and number of cysteine residues, the two active proteins were reduced and

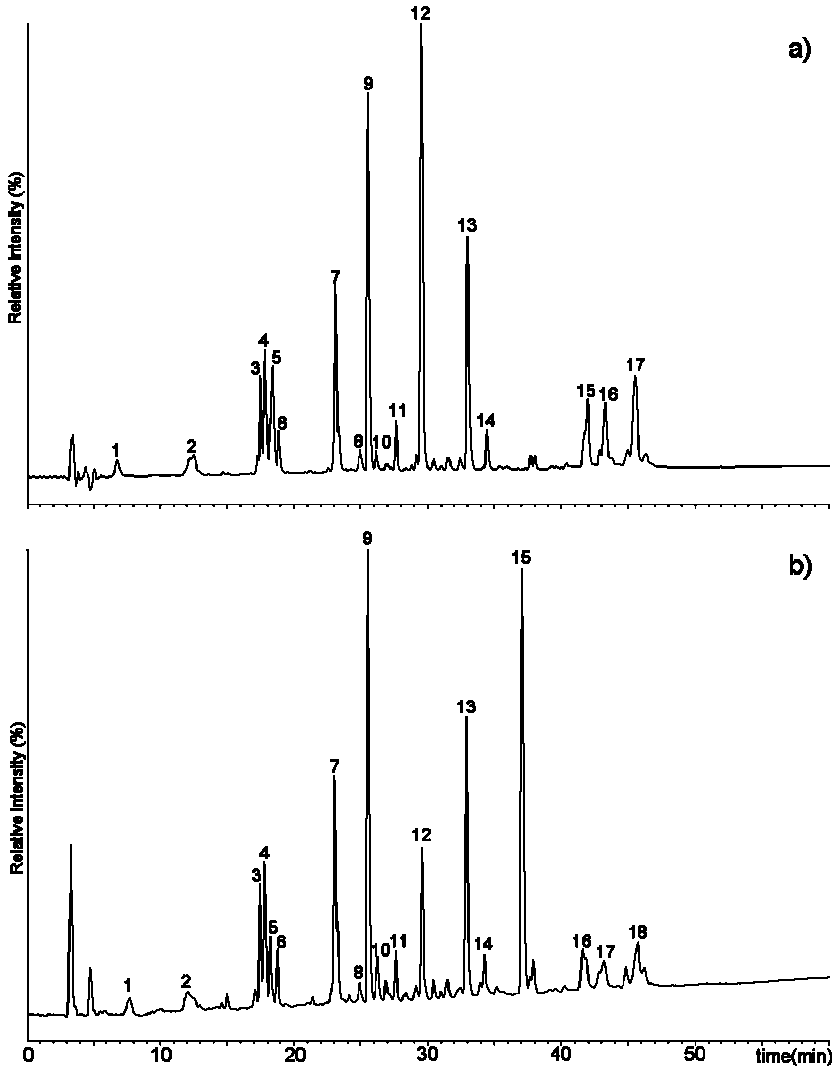

Fig. 3. RP-HPLC of the peptides obtained by tryptic digestion of the alkylated protein eluted in peak LmTX-I (panel a) and LmTX-II (panel b) shown in Fig. 1c.

alkylated as described in Section 2.6. The mass of the protein in peak LmTX-I after alkylation was 15,057.9 Da (Fig. 2b); the mass increasing of 812 Da indicated the presence of 14 Cys-CAM modified residues. The same increase in mass after alkylation was also registered for the protein in peak LmTX-II (mass of 14999.3 Da) as is shown in panel d, Fig. 2.

The same catalytic activity shown by LmTX-I and LmTX-II and the presence of the same number of cysteine residues indicate that these two proteins could be two isoforms of the same protein. The molecular mass difference between proteins contained in peaks LmTX-I and II implies that a single amino acid residue substitution P→R might have occurred accounting for a difference of 59 Da. Since the molecular mass difference might also depend on multiple amino acid substitutions, it was necessary to determine the primary structure of those proteins.

The two alkylated proteins were digested separately with trypsin, and the resulting tryptic peptides were fractionated by RP-HPLC (Fig. 3a and b). The chromatogram obtained shows similar profiles, except for the peak 15 eluted in LmTX-II alkylated tryptic digest (Fig. 3b). Each peak numbered in the two chromatograms shown in Fig. 3 was manually collected, lyophilised and "de novo" sequencing of the peptide was done by ESI-Q-TOF/MS/MS. Isoleucine and leucine residues were not discriminated in any of the sequences reported since they were indistinguishable in the low-energy CID spectra. Due to the external calibration applied to all spectra, it was also not possible to resolve the 0.036 Da difference between the glutamine and lysine residues, except for the lysine that was deduced based on the cleavage and missed cleavage of the enzyme.

The deduced sequence and measured masses of alkylated peptides of LmTX-I and LmTX-II are summarized in Table 1; on the basis of sequence determination, 19 peptides were find in the two proteins. The amino acid substitution P→ R according for mass difference of 59 Da, measured between molecular weights of LmTX-I and II intact proteins, was found in position 65. As shown in Table 1, peptides eluted in fractions 12 and 5 of LmTX-I, having the sequence "W D I/L Y R" (tandem MS spectra shown in Fig. 4a) and "Y S I/L K", respectively, are present only in LmTX-I alkylated digest, while the peptide generated from the trypsin secondary cleavage on Tyr_{66}, eluted in peak 15 of Fig. 3b, having a sequence of "W D I/L Y P Y" (tandem MS spectra shown in Fig. 4b), is present exclusively in the LmTX-II digest.

The sequence of each peptide was then submitted separately to the NCBI database using the protein search program BLAST-p. Using the position matches of the "de novo" sequenced peptides with homologous proteins present in the database, it was possible to deduce their original position on the unknown protein LmTX-I and LmTX-II [14].

Table 1
Measured molecular masses and deduced amino acid sequences of tryptic peptides obtained from the aklylated protein in peak LmTX-I and LmTX-II

LmTX-I HPLC fraction	Measured mass (Da)	Amino acid sequence	Measured mass (Da)	LmTX-II HPLC fraction	Theoretical mass (Da)
1	965.39	C R G P S E T C	965.40	1	965.37
2	551.28	F E T R	551.29	2	551.27
3	697.40	S I/L S T Y K	697.38	3	697.36
4	884.41	S G Y I/L T C G K	884.43	4	884.41
5	509.29	Y S I/L K	–	5	509.28
	691.36	C G W G G R	–		691.29
	853.58	R S I/L S T Y K	853.51		853.47
6	1504.56	C C F V H D C C Y G K	1504.59	6	1504.54
	817.42	V A A E C I/L R	817.43		817.41
7	1071.98	G C Y C G W G G R	1072.09	7	1071.40
8	914.38	M F Y P D S R	914.43	8	914.40
9	1741.70	G T W C E E K/Q I/L C E C D R	1741.69	9	1741.65
10	1234.48	Y G C Y C G W G G R	1234.52	10	1234.47
11	923.51	M I/L K F E T R	923.49	11	923.49
12	751.38	W D I/L Y R	–	12	751.37
	898.51	H I/L I/L K/Q F N K	898.52		898.50
13	1297.56	Y G Y M F Y P D S R	1297.56	13	1297.54
14	1615.69	Y A F Y G C Y C G W G G R	1615.68	14	1615.63
	–	W D I/L Y P Y	855.40	15	855.38
15	1612.79	K N A I/L P F Y A F Y G C Y	1612.79	16	1612.74
	2286.09	K N A I/L P F Y A F Y G C Y C G W G G R	2286.06		2286.09
16	2157.98	N A I/L P F Y A F Y G C Y C G W G G R	2157.98	17	2157.98
17	1887.86	N A I/L P F Y A F Y G C Y C G W	–	18	1887.78

C = alkylated cysteine.
The peptides were separated by RP-HPLC (Fig. 3a and b) and sequenced by mass spectrometry. Lysine residues shown in bold were deduced based on the cleavage and missed cleavage by trypsin. All molecular masses are reported as monoisotopic.

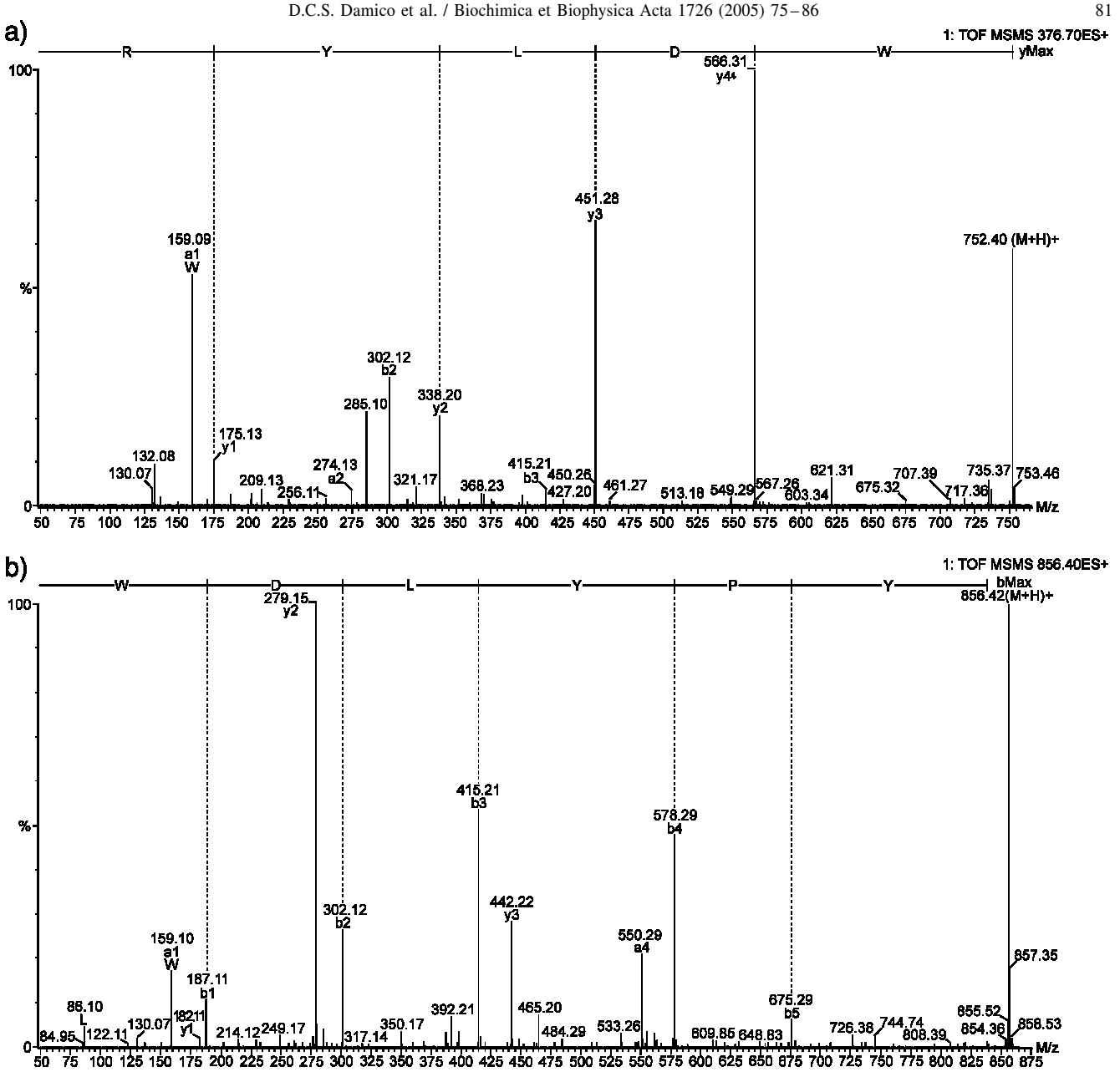

Fig. 4. ESI-QTOF-MS/MS spectrum of the tryptic peptides containing the amino acid substitution (LmTX-I) $R_{65} \longrightarrow$ (LmTX-II) P_{65} differentiating the two phospholipase A_2 isoforms. (a) y series distribution of the 5-residue-long tryptic peptide eluted in fraction 12 of the RP-HPLC profile of LmTX-I digest shown in Fig. 3a. (b) b series distribution of the 6-residue-long peptide eluted in fraction 15 of the RP-HPLC profile of LmTX-II digest shown in Fig. 3b.

The sequence of those proteins returns high homology with the sequence of a phospholipase A_2 present in the venom of *Crotalus scutulatus scutulatus* (Mojave rattlesnake) (PA2B_CROSS Accession Number P62023). The partial protein sequence obtained was then resubmitted to BLAST-p, with the search restricted to *Crotalinae* snakes. Fig. 5 shows the result of BLAST alignment between LmTX-I and LmTX-II with the phospholipase A_2 from *C. scutulatus scutulatus*.

The sequence coverage was 86% and 84% for LmTX-I and LmTX-II, respectively; both proteins shared the conserved sequence domains common to this group of proteins, including the 14 cysteines, the calcium-binding site located on Gly_{29}, Gly_{31}, Tyr_{27} and Asp_{48}, together with the amino acid of active site His_{47} and Asp_{89} (SwissProt database http://br.expasy.org/). The tandem mass spectra shown in Fig. 6, relative to the peptide eluted in fraction 6 of both digest, having the sequence C C F V H D C C Y G K, allows to classify both enzymes as D_{49} PLA_2.

The molecular homogeneity and basic nature of LmTX-I and LmTX-II were confirmed by two-dimensional electro-

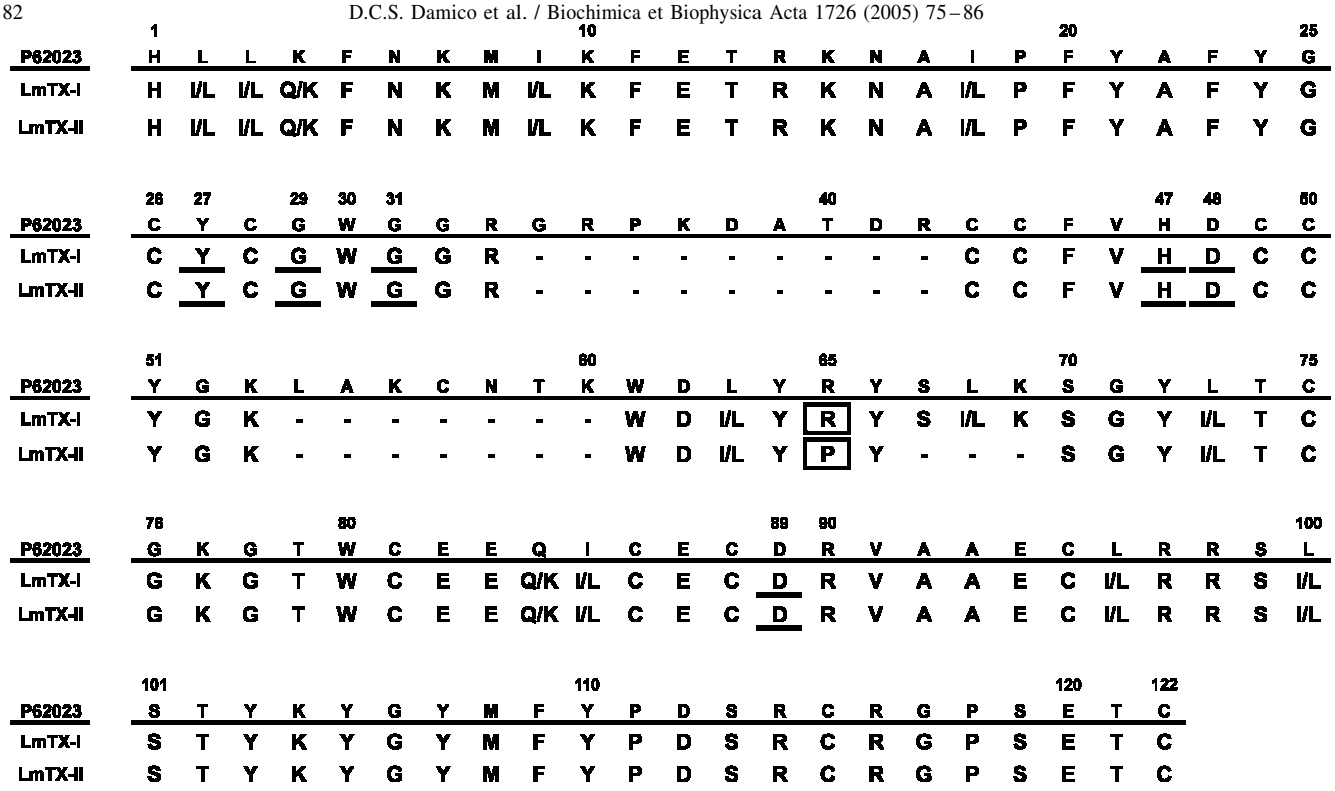

Fig. 5. Alignment of the deduced amino acid sequence of the two new phospholipase A_2 identified (LmTX-I and LmTX-II) with phospholipase A_2 presents in venom of *C. scutulatus scutulatus* (Mojave rattlesnake) (Accession Number P62023). Non-determined amino acid residues are indicated by (–); boxed amino acid residues differentiating LmTX-I from LmTX-II, underlined amino acid residues belong to phospholipase A_2 conserved domain. Leucine and isoleucine residues are indicated as I/L and glutamine and lysine are shown as Q/K. Lysine residues shown in bold were deduced based on the cleavage and missed cleavage by trypsin.

phoresis that showed a single protein spot with an estimated pI of 8.7 and 8.6, respectively (data not shown).

The PLA_2 activity of L. muta muta venom, FIII fraction, LmTX-I and LmTX-II were examined using the synthetic substrate 4-nitro-3-(octanoyloxy) benzoic acid [11]. The PLA_2 activity of the purified isoforms was higher than that of the venom and FIII (Fig. 7a). Under the conditions used, LmTX-I showed a discrete sigmoidal behaviour, mainly at low concentrations (Fig. 7b). Maximum enzyme activity occurred at 35–45 °C (Fig. 7c) and the pH optimum was 8.0 (Fig. 7d). The PLA_2 (LmTX-I) required Ca^{2+} for full activity. The activity was present with only 1 mM Ca^{2+} (Fig. 7e); however, the addition of Mn^{2+}, Mg^{2+}, Zn^{2+} or Cu^{2+} (10 mM) resulted in marked inhibition. The replacement of 10 mM Ca^{2+} by 10 mM Zn^{2+} or Cu^{2+} reduced the activity to levels similar to those seen in the absence of Ca^{2+} (Fig. 7e).

The effect of crotapotin (a non-toxic and non-enzymatic acid polypeptide naturally complexed with phospholipase A_2) in affecting the enzymatic activity of the PLA_2(s) has been investigated. All isolated crotapotins so far inhibited the activity of PLA_2 from different sources, but this inhibition is not uniform because it depends on the PLA_2 used. The main crotapotin isoform (F3) from *C. durissus cascavella* venom inhibited significantly LmTX-I by approximately 50% (Fig. 7f).

4. Discussion

The presence of PLA_2 isoforms in crotalinae snake venoms has been demonstrated by several authors and discussed by Valiente et al. [15]. Fuly et al. [4] reported the occurrence of a complex pattern of proteins in *L. muta* venom that eluted at different ionic strengths from a DEAE-cellulose column, some of which displayed indirect hemolytic activity. Fortes-Dias et al. [16] confirmed the high PLA_2 activity of *L. muta muta* venom and, by the inhibition produced by a PLA_2 inhibitor isolated from the plasma of another snake (*C. d. terrificus*), demonstrated the presence of five isoforms of PLA_2 (four acidic and one basic) in this venom.

We have isolated two basic D49 PLA_2 isoforms from *L. muta muta* venom using a simple and rapid procedure based on molecular exclusion chromatography on Superdex 75 and RP- HPLC. The use of NH_4HCO_3 (with Superdex 75) and acetonitrile (RP-HPLC) as the buffer system has the advantage that these solvents are easily eliminated by lyophilization, thereby eliminating the need for desalting in the case of ammonium bicarbonate.

The amino acid sequence of LmTX-I and LmTX-II showed a high level of homology with D49 PLA_2 such as Mojave toxin from *C. scutulatus scutulatus* venom (PA2B_CROSS accession number P62023), crotoxin from *C. d.*

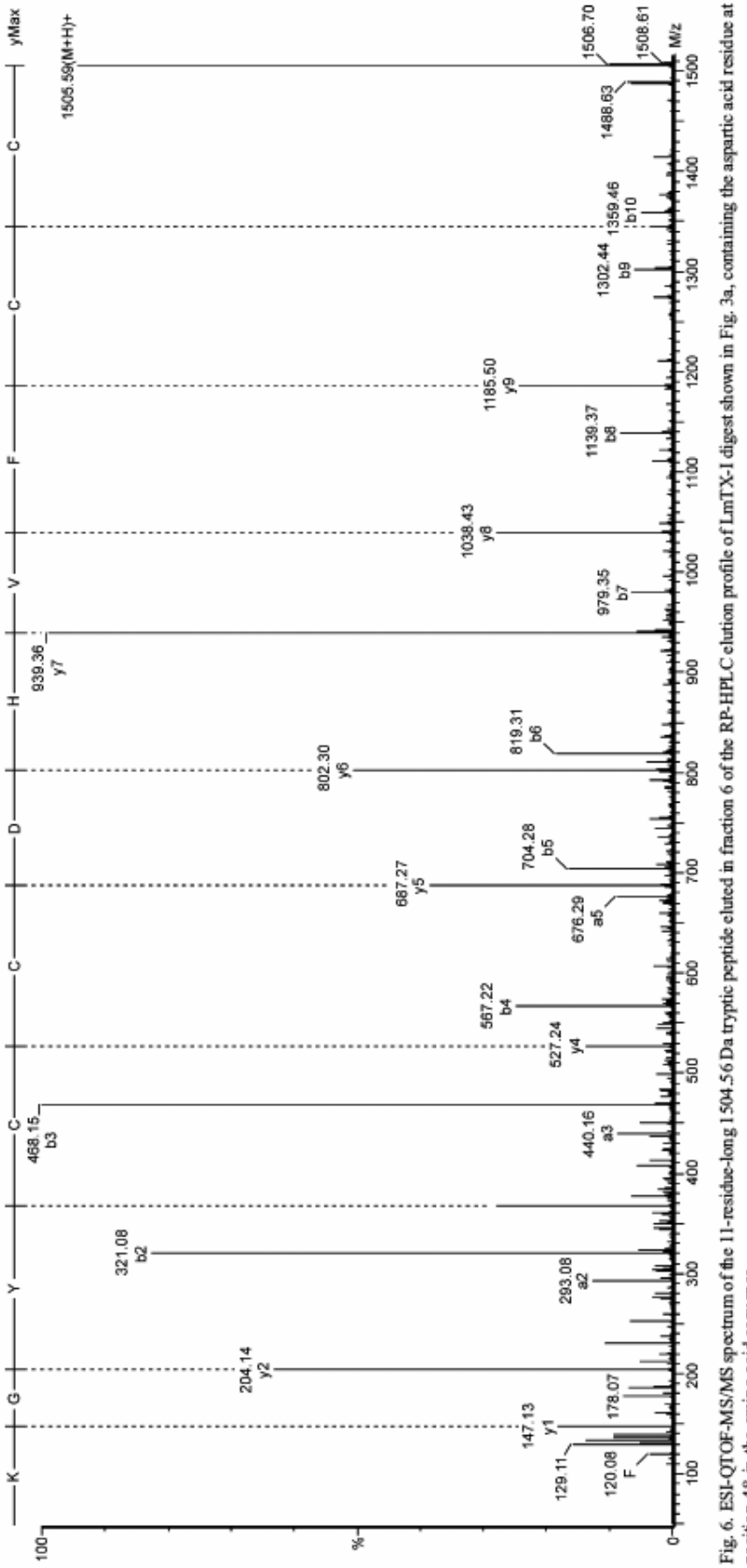

Fig. 6. ESI-QTOF-MS/MS spectrum of the 11-residue-long 1504.56 Da tryptic peptide eluted in fraction 6 of the RP-HPLC elution profile of LmTX-I digest shown in Fig. 3a, containing the aspartic acid residue at position 48 in the amino acid sequence.

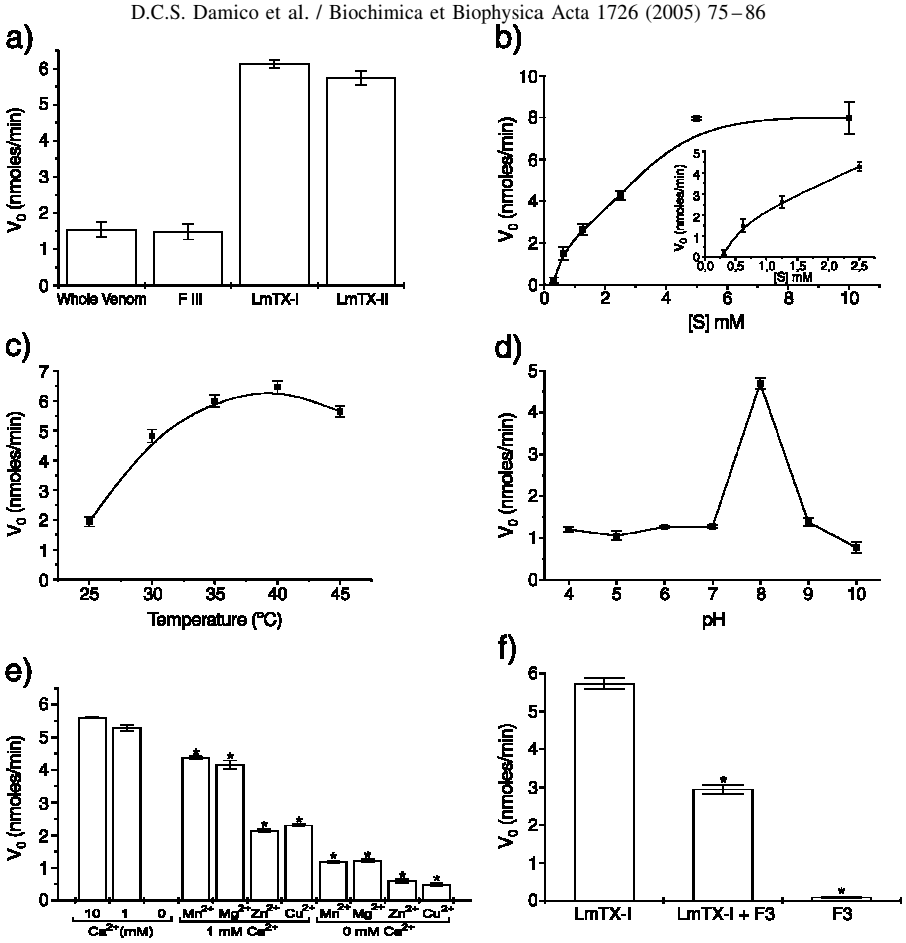

Fig. 7. (a) PLA_2 activity of *L. muta muta* venom, Fraction III, LmTX-I and LmTX-II. (b) Effect of substrate concentration on the kinetics of LmTX-I (PLA_2) activity. The inset shows the curvilinear shape at low substrate concentration. (c) Effect of temperature on the PLA_2 activity of LmTX-I. (d) Effect of pH on LmTX-I activity. (e) Influence of ions (10 mM each) on PLA_2 activity in the absence or presence of 1 mM Ca^{2+}. *$P<0.05$ compared to LmTX-I activity in the presence of 10 mM Ca^{2+}. (f) Inhibition of LmTX-I activity by crotapotin (F3) isolated from *C. durissus cascavella* venom. The results of all experiments are the mean ± S.E. of three determinations. *$P<0.05$ compared to LmTX-I activity.

terrificus venom (PA2C_CRODU, accession number P24027) and agkistrodotoxin from *Agkistrodon halys pallas* venom (PA23_AGKHP, accession number P14421). These toxins are β-neurotoxins that block neuromuscular transmission by a presynaptic action.

Secretory phospholipases A_2 ($sPLA_2$) are classified into three groups according to their primary structure. Group I PLA_2s is composed of both mammalian pancreas and snake venoms belonging to the Elapidae and Hydrophidae families, whereas group II PLA_2 is formed by venoms from Crotalidae and Viperidae families. Group III PLA_2s have mainly been isolated from bee (*A. mellifera*) and lizard venoms [6].

Despite their diversity of sources, three regions in class I and II PLA_2s retain a significantly high degree of amino acid-sequence homology. These regions contribute to the formation of the highly conserved secondary and tertiary structural elements including the N-terminal helix (residues 1–12), calcium binding (residues 25–33) and active site regions together with the amino acids forming the "hydrophobic channel" which binds the fatty-acyl chains of the phospholipid. The regions displaying a lower degree of amino acid homology correspond to structurally less conserved elements, and are likely determinants of the diverse pharmacology effects exhibited by venom PLA_2s [17].

The two proteins (LmTX-I and II) were differentiated from each other by a single amino acid substitution, Arg_{65} (LmTX-I) ⟶ Pro_{65} (LmTX-II). Arising from the distal tip of the first antiparallel helix of class I $sPLA_2$s is a distinctive loop (the "elapid" loop) of surface-exposed residues (region 55–67). This loop is absent in the class II enzymes, moderately developed among the class I elapids, and most prominent among the class I enzymes from exocrine pancreas ("pancreatic" loop) [18].

The residue 65 shows conserved among class I of secretory phospholipase A_2. In this position can be found residue L or V, which contributes to interfacial adsorption surface [18]. However, in class II PLA_2s, this residue presents variability. In the D49 PLA_2 family, it is not conserved [19,20]. Among the K49 PLA_2s, residue 65 is semiconservative, and most of them present in this position the S residue. The presence of residue R (65) in LmTX-I and P (65) in LmTX-II confirms this molecular hetero-

geneity, and shows that only the mutation of this residue doesn't prevent the catalytic activity (Fig 7a); other residues contribute to interfacial adsorption surface in the interfacial catalysis, like residues 3 (hydrophobic), 18 (hydrophobic), 19 (hydrophobic) and 21 (F-Y) [18].

All the described PLA_2s are highly stable and resistant to heat, acid, and urea, but catalytic activity is inactivated at high pH. When micellar substrates are used, maximum catalytic activity occurs at pH 7–8 and 30–55 °C [11,21–23]. LmTX-I showed maximum enzyme activity at 35–45 °C and greatest activity at around pH 8.0. The PLA_2 from *C. d. terrificus* venom is a typical PLA_2, since it hydrolyzes synthetic substrates at position 2 and preferentially attacks substrates in their micellar state [21]. They can hydrolyze phospholipids in monomeric, micellar or lipid bilayer phases. PLA_2 enzymes exhibit a large and abrupt increase (up to 10,000-fold) in their catalytic activity when monomeric phospholipids aggregate forms micelles at their critical micellar concentration [24]. This is due to the higher efficiency of interfacial catalysis, which depends on the absorption of the enzyme onto the lipid–water interface, strongly promoted by the presence of anionic amphipatic molecules within the membrane [25]. With synthetic substrate, LmTX-I behaved allosterically, especially at low substrate concentrations, which is in agreement with the results obtained by Bonfim et al. [26] for the PLA_2 of *Bothrops jararacussu* venom and Toyama et al. [27] for the F17 PLA_2 isoform purified from *C. d. terrificus* venom. Using the same synthetic non-micellar substrate, Rigden et al. [28] also observed that the dependence of activity on substrate concentration was markedly sigmoidal for the Piratoxin III from *Bothrops pirajai*.

A strict requirement for Ca^{2+} is characteristic of some PLA_2 [6,7]. LmTX-I showed typical Ca^{2+}-dependent PLA_2 activity like other D49 PLA_2 [29], and this activity was lower in the presence of other cations. Beghini et al. [12] also observed the same for PLA_2 from C. durissus cascavella venom.

The toxic crotalus phospholipase A_2 is considered a protein homologous to other phospholipases as well as to several presynaptic neurotoxins [30]. The basic crotalus phospholipase (Crotoxin B) binds to the acidic polypeptide crotapotin (Crotoxin A). The resulting phospholipase–crotapotin complex resembles the naturally occurring crotoxin. Crotapotin potentiates the toxicity and inhibits the enzymatic activity of the crotalus phospholipase A_2 [31]. Crotapotin (F3) from *C. durissus cascavella* inhibited significantly the catalytic activity of LmTX-I by approximately 50%. Our results are in agreement with the finding by Landucci et al. [32], who reported that highly purified crotapotin can inhibit pancreatic, bee, and other snake venom PLA_2, and Bonfim et al. [26] who reported that crotapotins from *C. d. terrificus* (F7), *C. d. collilineatus* (F3 and F4) and *C. durissus cascavella* (F3 and F4) decreased the catalytic activity of BJ IV (PLA_2 from *B. jararacussus*) by 50%. Together, these results suggest that crotapotin may bind to PLA_2 from *L. muta muta* venom in a similar manner to that for bothropic and crotalic PLA_2.

In the crotoxin complex, the enzyme active site is functional and can bind a monomer upon collision with phospholipid vesicles [33]. However, since it is defective in the ability to interact with the interface, and consequently, the "interfacial activation" observed with crotalus phospholipase (CB) will not occur with the crotoxin complex, the interaction of the crotoxin B with lecithin–water interfaces may require the exposure of a specific area on the enzyme surface, different off the active site and shielded in the crotoxin complex. This could explain why the rate of hydrolysis with the crotoxin complex is slower than with phospholipase (CB) and is poorly affected by the change in physical state of the phospholipid [34].

Thus, other studies are necessary to improve our knowledge of the properties of PLA_2s. One approach may be searching for the amino acid residues specifically involved in their actions. The 3-D structure of PLA_2s may lead to new insights to establish a better correlation in the structure–function relationships on the various biological effects of these enzymes.

Acknowledgements

This paper was supported by CAPES (Conselho de Aperfeiçoamento de Pessoal de Ensino Superior), FAPESP (Fundação de Amparo à pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). The authors thank to Beghini, D.G. for to supplying Crotapotin isoform. The authors thank to P.A. Baldasso for technical assistance.

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Functional characterization of a basic D49 phospholipase A_2 (LmTX-I) from the venom of the snake *Lachesis muta muta* (bushmaster)

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Abstract

Recently, we showed that the whole venom of *Lachesis m. muta* snake venom at low concentration (2 μg/ml) exhibited potent neurotoxicity, and myotoxicity was patent only at concentrations higher than 10 μg/ml. We have now examined these toxic activities of a basic phospholipase A_2, LmTX-I, isolated from the whole venom. LmTX-I caused a significant concentration dependent neuromuscular blockade in the BCp. The time to produce 50% neuromuscular blockade was 14.7 ± 0.75 min (30 μg/ml), 23.6 ± 0.9 min (10 μg/ml), 34 ± 1.7 min (2.5 μg/ml) and 39.2 ± 3.6 min (1 μg/ml), (n = 5/concentration; $p < 0.05$). Complete blockade with all tested concentrations was not accompanied by inhibition of the response to ACh. At the highest concentration, LmTX-I (30 μg/ml) significantly reduced contractures elicited by exogenous KCl (20 mM), increased the release of creatine kinase (1542.5 ± 183.9 IU/L *vs* 442.7 ± 39.8 IU/L for controls after 120 min, $p < 0.05$), and induced the appearance of degenerating muscle fibers (~15%). Quantification of myonecrosis indicated 14.8 ± 0.8 % and 2.0 ± 0.4 %, with 30 and 10 μg/ml venom concentration, respectively, against 1.07 ± 0.4 % for control preparations. The findings indicate that the basic PLA_2 present on venom from *L. m. muta* (LmTX-I) possesses a dominant neurotoxic action on isolated chick nerve-muscle preparations, whereas myotoxicity was mainly observed at the highest concentration used (30 μg/ml). These effects of LmTX-I closely reproduce the effects of the whole venom of *Lachesis muta muta* in chick neuromuscular preparations.

Key words: Biventer cervicis preparation; Asp49 Phosphoplipase A_2; *Lachesis muta muta* venom; Myotoxicity; Neuromuscular blockade; Neurotoxicity

1. Introduction

Lachesis muta muta (bushmaster) is the largest Crotalinae snake in the world. It is widely distributed in the tropical rain forests of Central and South America, including Brazil. Clinical signs and symptoms of envenoming by bushmaster are nausea, vomiting, diarrhea and sweating. The symptoms at the bite site are pain and oedema, swelling, hemorrhage and coagulopathy, which are all similar to those caused by the bites of snakes of *Bothrops* genus and other genera of Latin American pit vipers (Jorge et al., 1997). Occasionally "neurotoxic signs" (divergent strabismus, dysarthria, dysphagia) are present (Diniz and Oliveira, 1992).

The venom of *L. m. muta* contains several components which are known to produce activating or inactivating effects against the haemostatic system and may also disrupt the integrity of vessel walls due to the action of hemorrhagic toxins (Estêvão-Costa et al., 2000). Metalloproteinases are largely responsible for the hemorrhagic syndrome in snakebite envenomation (Gutiérrez et al., 2005).

L. m. muta venom also possesses kininogenase activity that might explain the "neurotoxic signs" but no specific neurotoxic component (s) that affect neuromuscular transmission have been identified in the venom.

Recently, we reported neurotoxic and myotoxic activities in the venom of *L. m. muta* (Damico et al., 2005a) and it seems clear that the venom has pharmacologically active components that act on the neuromuscular junction and muscle fibers, whose intensity of action will depend on venom concentration and the type of nerve-muscle preparation (mouse or chick). We have purified and characterized a PLA_2 isoform from *L. m. muta* venom denominated LmTX-I (Damico et al., 2005b). This toxin is a basic

PLA_2 with pI 8.8 and a single polypeptide chain of 14254.4 Da. The primary structure of LmTX-I showed a high level of homology with Asp49 containing PLA_2 such as Mojave toxin from *Crotalus scutulatus scutulatus* venom; the basic PLA_2 from *Crotalus durissus terrificus* venom and Agkistrodotoxin from *Agkistrodon halys pallas* venom (Damico et al., 2005b). All these toxins are β-presynaptic-acting neurotoxins that block neuromuscular transmission. In this work, we used chick nerve-muscle preparations to assess the neurotoxicity and myotoxicity of LmTX-I.

2. Materials and Methods

2.1. Venom and toxin

The venom and the solvents (HPLC grade), 4-nitro-3 (octanoyloxy) benzoic acid, and other reagents were from Sigma Chemical Co. (St. Louis, MO, USA). The water, buffers and solvents used in the purification protocols were filtered through 0.22 μm filters (Millipore) and degassed by sonication. RP-HPLC was made using a μ-Bondapack C18 column (0.78 cm x 30 cm) and a Jones Chromatography Genesis C8 column (0.46 cm x 15 cm).

2.2. Animal

Male HY-LINE W36 chicks (4-8-day-old) were supplied by Granja Ito S/A (Campinas, SP, Brazil) and had free access to food and water. All procedures were done in accordance with the general guidelines proposed by the Brazilian Council for Animal Experimentation (COBEA), protocol 677-1.

2.3. Purification procedure

Lachesis m. muta venom (35 mg) was dissolved in 0.3 ml of ammonium bicarbonate buffer (1.0 M, pH 8.0) and centrifuged at 15,000 x g for 5 min. The sample was then applied onto a Superdex 75 (1 cm x 60 cm) column 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. The elution profile was monitored at 280 nm and the fraction containing PLA_2 activity was pooled, lyophilized, and stored at -20ºC. Five milligrams of the PLA_2 fraction was dissolved in 200 µl of 0.1% (v/v) trifluoroacetic acid (solvent A). The resulting solution was clarified by centrifugation and the supernatant was applied to a µ-Bondapack C18 column (0.78 cm x 30 cm; Waters 991- PDA system). Fractions were eluted using a non-linear gradient (0-54%, v/v) of acetonitrile (solvent B) at a constant flow rate of 1.0 ml/min over 40 min and the resulting fractions were manually collected. Fractions containing protein species, active against the PLA_2 assay, were submitted to a new RP-HPLC purification step performed with a Jones Chromatography Genesis C8 column (0.46 cm x 15 cm), with a linear gradient from 20-35% B over 90 min at a constant flow rate of 1.0 ml/min. The elution profile of both analyses was monitored at 220 and 280 nm, and the collected fractions were lyophilised and conserved at –20ºC.

2.4. Electrophoresis in SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the fractions was done according to Laemmli (1970), using a 15% separating gel and a 5% stacking gel under reducing (with 1 M DTT) and nonreducing conditions.

2.5. PLA_2 activity

PLA_2 activity was measured using the assay described by Cho and Kézdy (1991) and Holzer and Mackessy (1996), modified for 96-well plates (Beghini et al., 2000). The standard assay mixture contained 200 µl of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 µl of substrate, 20 µl of water, and 20 µl of PLA_2 in a final volume of 260 µl. After addition of PLA_2 (20 µg), the mixture was incubated for up to 40 min at 37ºC, with the absorbance being read at 10 min intervals. The enzyme activity, expressed as the initial velocity of the reaction (V_0), was calculated based on the increase in absorbance after 20 min. The assay was done in triplicate and the absorbances at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA).

2.6. Chick biventer cervicis muscle preparation (BCp)

Male chicks (4–8-day-old) were killed with halothane inhalation and the biventer cervicis muscles were removed (Ginsborg and Warriner, 1960) and mounted under a tension of 1.0 g in a 5 ml organ bath containing warmed (37 ºC), 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 (Load Cell BG- 10GM kulite Semicondutor Products, Inc, NJ, USA) coupled to a Gould RS3400 recorder. The BCps were allowed to stabilize for

at least 20 min before the addition of ACh or KCl, and concentrations (30, 10, 2.5 or 1 μg/ml) of LmTX-I were applied (*n* = 5). Control experiments were done using Krebs solution. Contractures to exogenously applied acetylcholine (ACh, 110 μM for 60 s) and KCl (20 mM for 120 s) were obtained in the absence of field stimulation prior addition of LmTX-I and at the end of the experiment.

2.7. Creatine kinase activity

Electrically stimulated BCps were incubated with LmTx-I (30 or 10 μg/ml) as described above (*n* = 5). At 0, 15, 30, 60, 90 and 120 min incubation, samples of the bath solution (30 μl) were collected and immediately stored at 4ºC. Creatine kinase (CK) activity was measured using commercial kit (Bioclin, Labcenter, Campinas, S. P., Brazil). The original volume of the incubation bath (5 ml) was always maintained by replacing each of the five 30 μl aliquots collected, by an equal volume of freshly prepared Krebs solution. Enzyme activity was expressed as international units/liter (IU/l), and used as a quantitative biomarker to evaluate (biochemically) fiber damage, and hence LmTX-I myotoxic potency.

2.8. Morphological and morphometrical analysis

After complete neuromuscular blockade of BCp incubated with 10 or 30 μg/ml of LmTX-I, the preparations (*n* = 5) were fixed in Bouin's solution for 24–48 h (control preparations were incubated in Krebs solution for 120 min, n = 5), followed by washing with 5% ammonium hydroxide aqueous solution. The muscles were then dehydrated in ethanol series and embedded in historesin. After resin polymerization (60ºC), the blocks

of muscle were sectioned with a microtome (Leica model RM 2145, Leica Microsystems Ltd, Heerbrugg, Switzerland). Sections (2 μm thick) were stained with toluidine blue and examined by light microscopy using an Olympus microscope (Olympus Optical Co. Ltd, Tokyo, Japan) prior to photographing. The extent of muscle damage was assessed quantitatively by counting 70 fibers (normal or damaged) in four non-overlapping randomized areas in a total of 280 fibers per preparation. BCps incubated with 10 or 30 μg/ml of LmTX-I (n = 5) had a total of 1400 fibers counted. The same procedure was done for the control preparations (n = 4; 1120 fibers counted). The percentage of damaged fibers was calculated.

2.9. Statistical analysis

Each experimental protocol (twitch tension records, CK and muscle fibers counting) was repeated at least five times, and the results reported as the mean ± SEM. Anova followed by Tukey-Kramer multiple test was used for statistical comparison of the data. The $p < 0.05$ value was considered to indicate significance.

3. Results

3.1. Purification

Fractionation of *L. m. muta* venom by molecular exclusion chromatography on Superdex 75 resulted in seven main fractions (I - VII) (Fig. 1a), of which peak III had PLA_2 activity and was chromatographed on an analytical RP-HPLC C18 column. Only one of the five main fractions had PLA_2 activity (Fig. 1b) corresponding to LmTX. The fig. 1c shows the result of the repurification of active LmTX fraction by RP-HPLC using a C8 column; the chromatogram shows the presence of five main peaks, among these,

chromatographic peak denominated LmTX-I contained a single protein. SDS-PAGE analysis of the toxin showed a single polypeptide chain of approximately 14 kDa under reducing and non-reducing conditions (Fig. 1c insert). Electrospray mass spectrometry analysis performed on LmTX-I peak confirmed the purity of the protein and determined the exact molecular mass of 14,254.4 Da (Damico et al., 2005b).

3.2. Neuromuscular blockade in chick biventer cervicis preparation (BCp)

All the tested PLA_2 concentrations (30, 10, 2.5 or 1 μg/ml) caused an irreversible concentration-dependent blockade of neuromuscular transmission (Fig. 2), which was sustained even after several washings. The time required for 50% twitch tension blockade was 14.7 ± 0.75 min at a toxin concentration of 30 μg/ml, 23.6 ± 0.9 min at 10 μg/ml, 34 ± 1.7 min at 2.5 μg/ml and 39.2 ± 3.6 min at 1 μg/ml (n = 5/dose). The twitch tension records of control preparations remained stable at 94% from 120 min of incubation in Krebs solution (n = 5).

3.3. ACh- and KCl-induced contractures in BCp before and after PLA_2 addition

At the highest concentration used (30 μg/ml), the PLA_2 significantly reduced the KCl- (20 mM) induced contractures when compared to the control values (Fig. 3). There was no inhibition of ACh-induced contractures when compared to control values. In the control preparations, the contracture like responses to ACh and KCl were stable for at least 120 min (data not shown).

3.4. Myotoxic activity of LmTX-I: CK activity *in vitro*

As shown in Figure 4, LmTX-I, the basic PLA_2 from *L. m. muta,* caused a concentration and time-dependent increase in CK activity in the bathing medium containing BCp, presumably reflecting the enhanced release of CK from the muscle preparations.

3.5. Myotoxic activity: Morphological changes

Chick BCp incubated with Krebs solution alone (controls) showed normal muscle morphology with regular muscle fiber fascicles and uniform intensity of cell staining (Fig. 5A). Preparations incubated with LmTX-I (10 μg/ml), despite maintaining well-organized muscle fascicles, exhibited a number of densely staining muscle fibers and hence appeared more stained (dark) usually considered to reflect regions where myofibrils are highly contracted (Fig. 5B). Muscles incubated with LmTX-I (30 μg/ml) showed a medial disorganization of the fascicles, an increased number of darkly staining cells, and in addition a few cells in early stages of degeneration (Fig. 5C). Muscle fiber counts showed that LmTX-I significantly damaged 14.8 ± 0.8 % of fibers when 30 μg/ml PLA_2 was used, whereas only 2.0 ± 0.4 % of the fibers were affected when 10 μg/ml was used in the incubation bath, which did not significantly differ from control values (1.07 ± 0.4%).

4. Discussion

The monomeric toxin LmTX-I is an example of the so-called type II Asp49 phospholipase A_2 (as distinct from the Lys49 phospholipases that are much lower in hydrolytic activity despite their structural similarity to their Asp49 homologues). LmTX-I

caused an irreversible concentration-dependent blockade of the indirectly elicited twitch responses of the BCp at concentration as low as 1 μg/ml, which was maintained even after washing the preparation over three times. At this concentration, there was no evidence of significant myotoxicity. The complete blockade of the contraction of the muscle was not accompanied by any inhibition of the responses to ACh. Thus, the neuromuscular blockade produced by LmTX-I may be attributed to presynaptic activity, either by blocking axonal conduction or by affecting transmitter release at the motor nerve-terminal.

Several type II Asp49 monomeric neurotoxic phospholipases have been well studied, like caudoxin (Viljoen et al., 1982), agkistrodotoxin (Kondo et al., 1989), ammoditoxin (Ritonja and Gubensek, 1985), and notexin (Halpert and Eaker, 1975). These are all typical examples of type II phospholipases, a large group of homologous polypeptides many of which are neurotoxic, causing neuromuscular paralysis by depleting the motor nerve terminal of transmitter (Kini, 2003; Harris et al., 2003).

At concentrations of LmTX-I of 30 μg/ml or more, LmTX-I caused a reduction in the response to KCl of about 30%, muscle fibre damage to approximately 15%, and a significant increase in the release of CK into the bathing medium. Thus, we conclude that at high concentrations LmTX-I is myotoxic. Taken together, morphological quantitative analysis and CK measurements indicate that LmTX-I is weakly myotoxic, i.e., does not induce extensive myonecrosis, and probably only in a parcel of the cells the permeabilization of the sarcolemma has occurred.

In conclusion, LmTX-I, an Asp49 basic PLA_2 from *Lachesis muta muta* (bushmaster) venom showed "*in vitro*" a potent neuromuscular pre-synaptic blocking

activity in chick nerve-muscle preparations, evident even at 1 μg/ml concentration, whereas comparatively it presents poor myotoxicity. The results suggest that LmTX-I is the main toxin responsible for the effects seen with the whole venom of this species, in which neurotoxic effect surpass the myotoxic one.

Acknowledgments

D. C. S. D. is a recipient from a Doctoral fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior). This work was also supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). The authors thank Mr. Gildo Bernardo Leite, Mr. Paulo Baldasso, Ms. Rosana de Andrade Silva and Mrs Marta Beatriz Leonardo for technical support.

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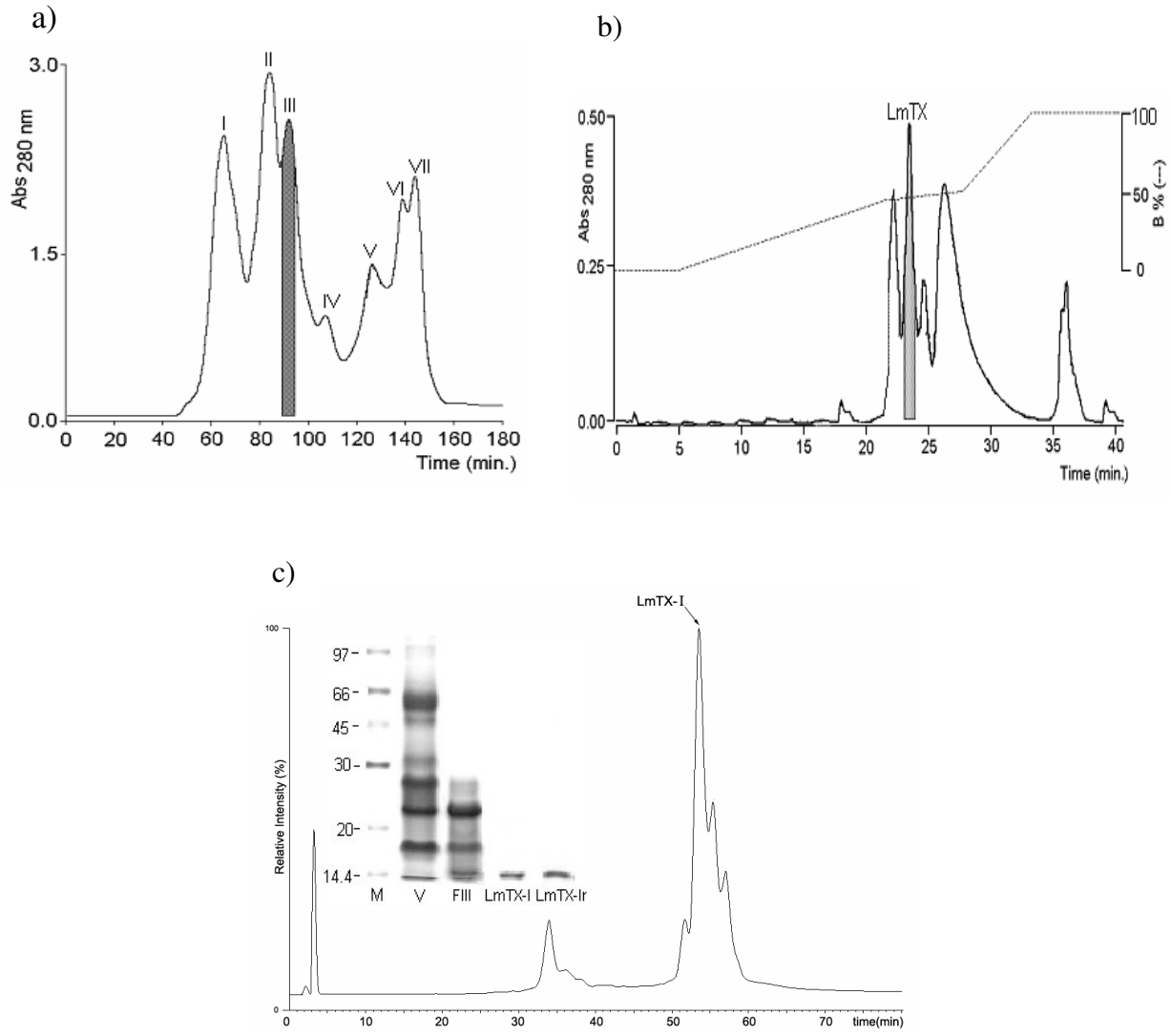
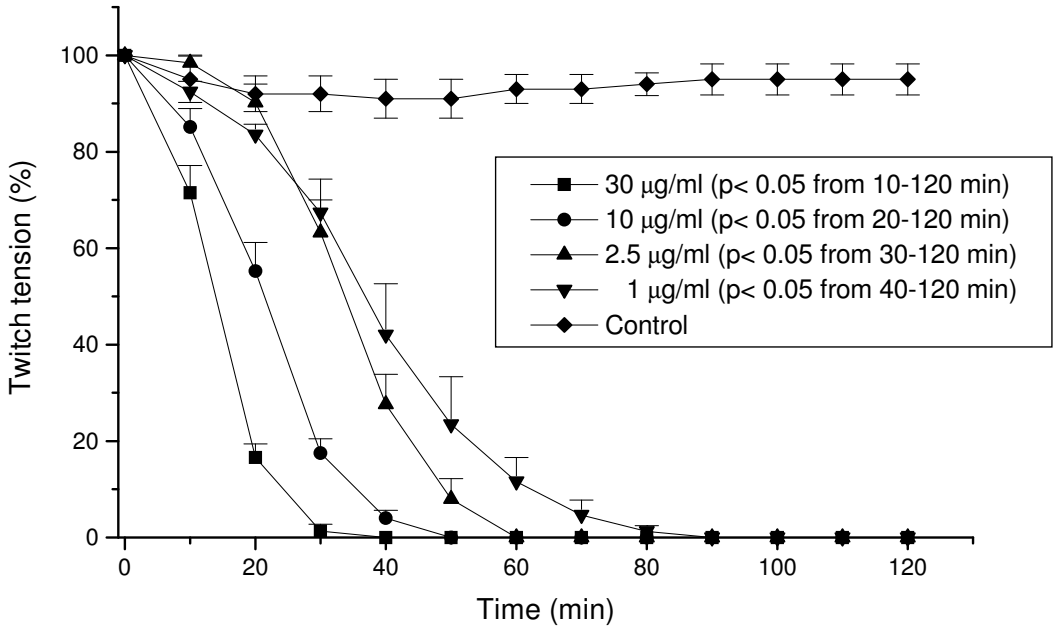


Figure 1. Daniela C. S. Damico, Lilian G. F. Bueno, Léa Rodrigues-Simioni, Sérgio Marangoni, Maria Alice da Cruz-Höfling and José Camillo Novello.



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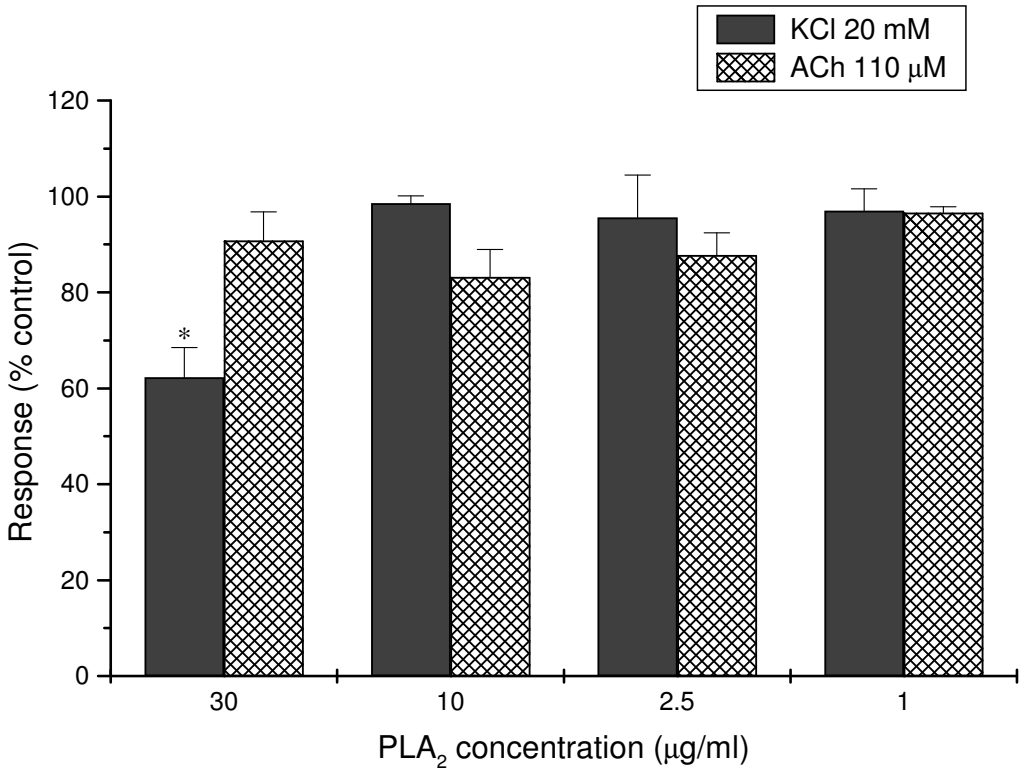

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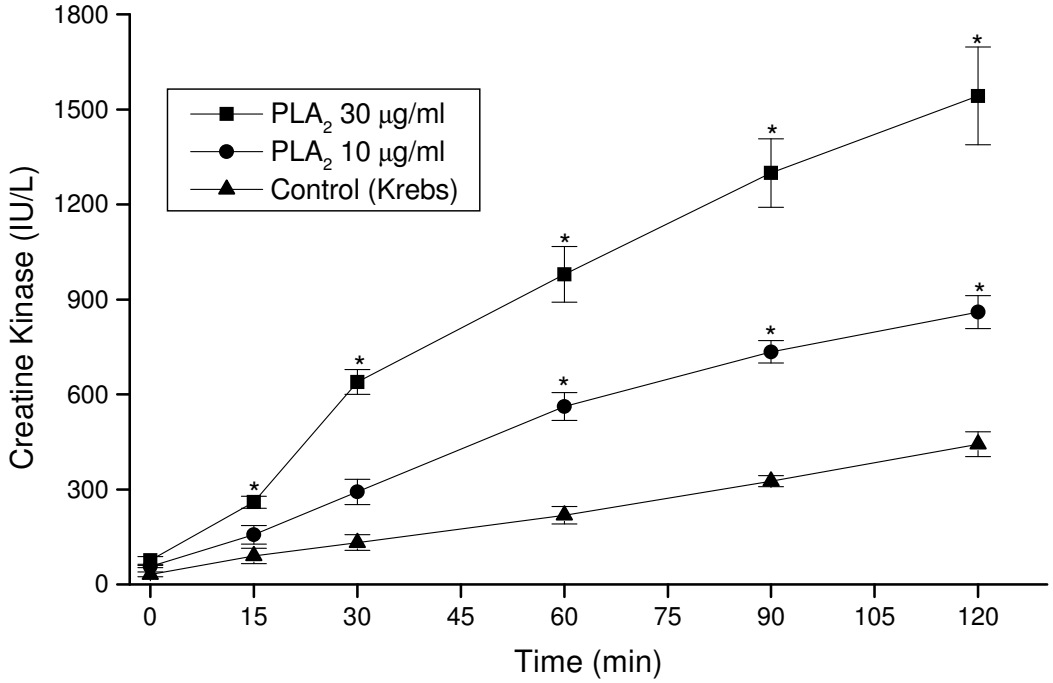

Figure 4. Daniela C. S. Damico, Lilian G. F. Bueno, Léa Rodrigues-Simioni, Sérgio Marangoni, Maria Alice da Cruz-Höfling and José Camillo Novello.

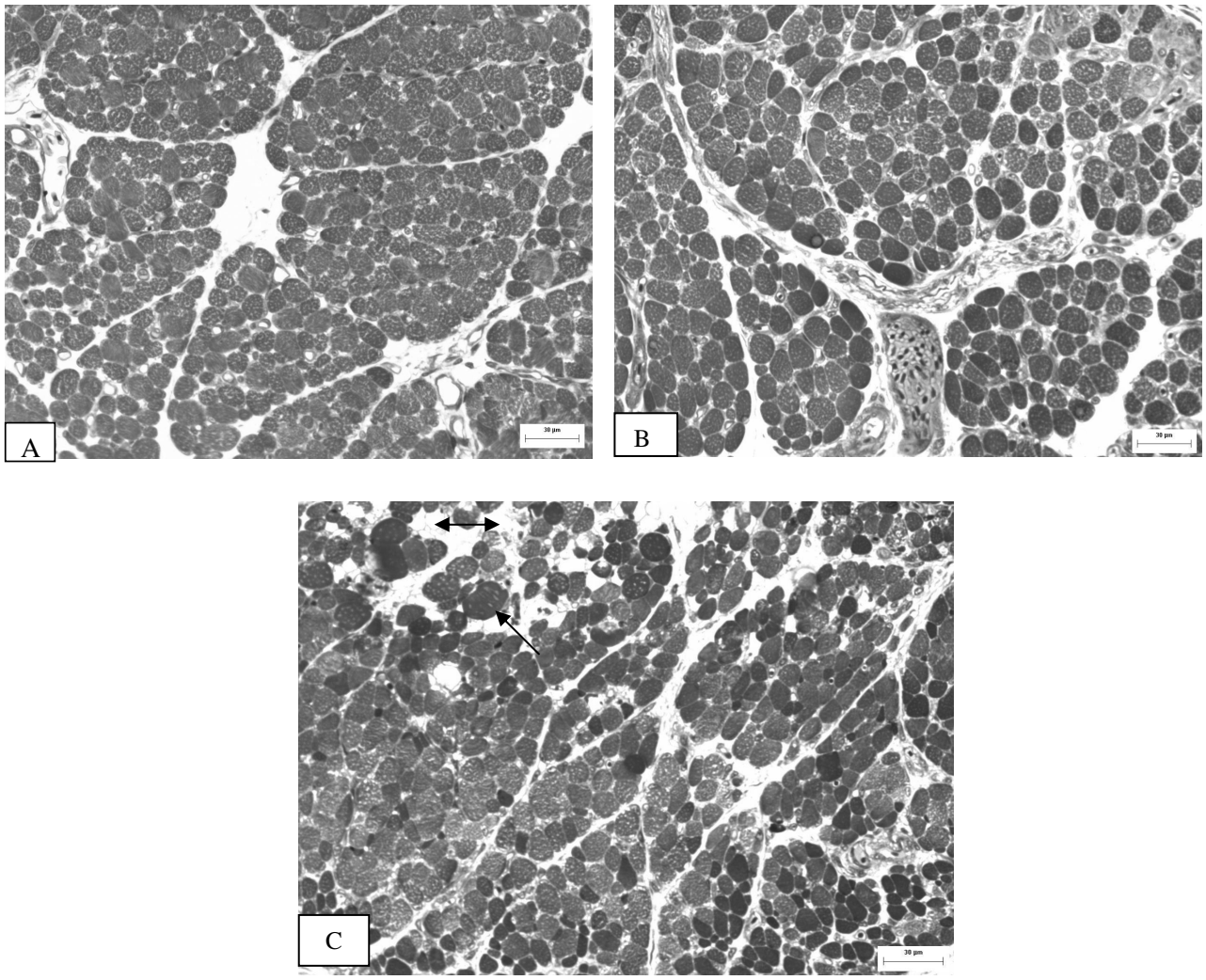

Figure 5. Daniela C. S. Damico, Lilian G. F. Bueno, Léa Rodrigues-Simioni, Sérgio Marangoni, Maria Alice da Cruz-Höfling and José Camillo Novello.

Legends for figures

Figure 1. (a) Elution profile of *Lachesis muta muta* venom by molecular exclusion chromatography on Superdex 75 column. Fraction III contained PLA_2 activity. (b) Elution profile of FIII following RP-HPLC on a μ-Bondapack C18 column. The peak corresponding to active fraction (LmTX) is indicated. (c) RP-HPLC profile of LmTX eluted on a Genesis (Jones chromatography) C8 column. The chromatographic peak contained PLA_2 active (LmTX-I) is indicated. Insert: electrophoretic profile in SDS-PAGE (12.5% gel). M- Molecular mass markers; V- *L. m. muta* venom; FIII- fraction from molecular exclusion chromatography; LmTX-I- PLA_2 from *L. m. muta,* and LmTX-Ir- PLA_2 from *L. m. muta* reduced.

Figure 2. Neuromuscular blockade induced by the PLA_2 (LmTX-I) from *Lachesis m. muta* on the chick biventer cervicis muscle preparation. Each point represents the average from five experiments ± SEM. $P < 0.05$ compared to control.

Figure 3. Effect of the PLA_2 from *Lachesis m. muta* on the response of the chick biventer cervicis preparation to the acetylcholine (ACh) and potassium (KCl). Each point represents the average from five experiments ± SEM. $*p < 0.05$ compared to control.

Figure 4. Creatine kinase (CK) concentration from chick BCp following incubation with LmTX-I (30 or 10 μg/ml). The venom was added to the bath at time zero and the CK activity was expressed as means ± SEM of five experiments. $*p < 0.05$ compared to control.

Figure 5. Light micrographs of chick BCp (cross-sections). (A) BCp incubated with Krebs solution alone after 120 min incubation (control), (B) BCp incubated with LmTX-I (10 μg/ml). Note the regular fiber fascicles, however, some cells exhibited more compacted myofibrils and hence appeared more stained. (C) BCp incubated with LmTX-I (30 μg/ml). Comparatively, the clusters of cross-sectioned myofibrils seen in control preparations were not visible in the preparations incubated with the toxin, therefore an increasing number of dark cells are seen (arrows). Only a few fibers exhibit marked damage (double arrows). Toluidine blue. Bar = 30 μm for all panels.

Study on the cytotoxic effect of the *Lachesis muta muta* crude venom and its purified basic PLA_2 (LmTX-I) in cultured cells

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ABSTRACT

In this work, we investigated the cytotoxicity of *Lachesis m. muta* venom and its purified phospholipase A_2 (PLA_2) LmTX-I in cultured Madin-Darby canine kidney (MDCK) and skeletal muscle (C2C12) cell lines. As revealed by neutral red dye uptake assay, the crude venom at concentrations of 10 and 100 μg/ml induced a significant decrease of approximately 25% and 60% in cell viability of MDCK cells, respectively, after 3 h of incubation. The PLA_2 isoform LmTX-I at the concentrations tested (10-270 μg/ml) displayed no cytotoxicity in both MDCK and in a skeletal muscle (C2C12) cell lines. However, 100 μg of LmTX-I/ml induced a steady but significant decrease in transepithelial electrical resistance (R_T) across MDCK monolayers after 3 h of incubation. Venom (100 μg/ml) also decreased the R_T across MDCK monolayers in approximately 30 % after 1 h and, at 3 h of incubation with the venom at this concentration, the R_T went down to nearly zero. Feulgen's reaction revealed chromatin condensation, apparent reduction in the number of mitotic nuclei and nuclear fragmentation of some MDCK cells after incubation with *L. m. muta* venom at the concentrations of 10 or 100 μg/ml. Monolayer exposure to crude venom (10 μg/ml) for 5 h and 24 h resulted in morphological changes such as the presence of groups of cells with a fusiform or a more rounded format as assessed by scanning electron microscopy. The staining with TRITC-labelled phalloidin, that reveals F-actin, showed a marked disarray of the stress fiber following *L. m. muta* crude venom exposure. In contrast, LmTX-I had no effect on nucleus and cell morphologies as well as on stress fiber organization. These results indicate that *L. m. muta* venom exerts toxic effects on cultured MDCK cells. The PLA_2 (LmTX-I) probably does not contribute to the direct venom cytotoxicity, these effects are probably mediated by metalloproteinases/disintegrins and other components of the venom.

Key words: *Lachesis muta muta*; snake venom; cytotoxicity; MDCK cells; phospholipase A_2.

1. Introduction

Snakes from the genera *Bothrops*, *Crotalus*, *Lachesis* and *Micrurus* are responsible for the majority of the snake accidents in Brazil. Clinical symptoms of *Bothrops* and *Lachesis* accidents share several similarities but differ from those induced by *Crotalus* and *Micrurus* bites (De Moura et al., 1998). Accidents induced by *Bothrops* and *Lachesis* are characterized by local pain, oedema, haemorrhage and necrosis at the site of the bite. Depending on the severity of the accident and sensitivity of the patient, systemic complications may also occur such as cardiovascular shock, coagulation dysfunction, haemolysis and renal failure (Rucavado et al., 1999).

Successful treatment of snakebites depends not only on prompt administration of specific antiserum but also on full knowledge of the biological effects induced by the snake venoms. Pharmacological and biochemical studies on Bothrops venom are relatively extensive (Lomonte et al., 1999; Collares-Buzato et. al., 2002; Angulo and Lomonte, 2005), probably because the incidence of the accidents with this snake is higher. In contrast, studies on the pharmacological action of *Lachesis muta muta* venom, the largest poisonous snake of Brazil, are scant.

Recently, we reported the neurotoxic and myotoxic activities *in vitro* of the venom from *L. m. muta* and that these activities were dose-dependent both in mouse and chick nerve-muscle preparations (Damico et al., 2005a). It became clear that the venom has pharmacologically active components that act on the neuromuscular junction and muscle fibers, whose intensity of action will depend on the venom concentration and on the type of nerve-muscle preparation (mouse or chick).

Phospholipases A_2 (PLA_2: EC 3.1.1.4) are major components of snake venoms that have acquired a variety of toxic activities during evolution, including myotoxic,

neurotoxic, cytotoxic, anticoagulant, and inflammatory effects (Ogawa et al., 1995; Kini, 1997). PLA_2s isolated from snake venoms have been classified as follows: those from species of the Elapidae family belong to group I, which also includes the pancreatic PLA_2s of mammals, whereas those from Viperidae are classified as group II, together with mammalian secreted inflammatory PLA_2s (Arni and Ward, 1996; Kudo and Murakami, 2002). Current evidence indicates that PLA_2 myotoxins act primarily on the sarcolemma, rapidly altering its permeability through either catalytically dependent or independent mechanisms (Dixon and Harris, 1996; Gutiérrez and Ownby, 2003; Lomonte et al., 2003). However, the nature of the membrane acceptor site(s) involved in these mechanisms, and the detailed molecular events that follow toxin binding are still unknown. Several of the group II myotoxins studied in cell culture models belong to the subgroup of Lys49 PLA_2 homologues, protein variants that are enzymatically inactive due to a number of amino acid substitutions, including the critical Asp49 to Lys49 change (reviewed by Lomonte et al., 2003). Among the group II PLA_2s evaluated, no major differences were observed in the cytolytic activity induced by either Asp49 variants, indicating that this effect can develop in the absence of an intrinsic enzymatic activity of the myotoxins (Lomonte et al., 1999).

Recently, we described that the basic Asp49 PLA_2 purified from the crude venom of *L. m. muta* (LmTX-I) possesses a preponderant neurotoxic action *in vitro* on chick nerve-muscle preparations (1 μg/ml), whereas myotoxicity was mainly observed at the highest concentration used (30 μg/ml) (Damico et al., 2006).

In the present work, we investigated a putative direct cytotoxic effect of the *L. m. muta* crude venom and its purified PLA_2 (LmTX-I) in cultured cells using a renal tubular

epithelial and a skeletal muscle (C2C12) cell lines. MDCK (Madin-Darby Canine Kidney) cells constitute a well-established cell line and have been often employed to investigate the cellular responses to toxic agents and venoms (Chan et al., 1989; Collares-Buzato et al., 2002; Schwerdt et al., 2004; Peixoto and Collares-Buzato, 2005). Herein, MDCK cells were used as a model for studying the generality of the toxic action of the *L. m. muta* venom and the LmTX-I.

2. Material and Methods

2.1. Venom and Reagents

The venom and the solvents (HPLC grade) and other reagents were supplied by Sigma Chemical Co. (St. Louis, MO, USA). The water, buffers and solvents used in the purification protocols were filtered through 0.22 μm filters (Millipore, Bedford, USA) and degassed by sonication. Sterile plastic material for cell culture was supplied by Corning, (Corning, NY, USA). Cell culture media and supplements were purchased from Cultilab (SP, Brazil).

2.2. Purification procedure

The PLA_2 from *Lachesis muta muta* (LmTX-I) utilized in this study was purified as described previously by Damico et al. (2005b).

2.3. Cell culture

Low-resistance MDCK renal epithelial cells were obtained from Adolfo Lutz Institute (São Paulo, Brazil). The cells were grown in plastic flasks at 37°C in a humidified atmosphere of 2% CO_2/air, with Eagle´s minimum essential medium (MEM)

supplemented with 10% fetal calf serum, 1% penicillin (10.000 UI/ml) and streptomycin (10 mg/ml) (Collares-Buzato *et al.*, 2002). The cells were passaged weekly after treatment with trypsin (2.5 mg/ml) and 0.2 % EDTA in a balanced salt solution without Ca^{2+} or Mg^{2+}. When required, cells were seeded at high density (0.5-1.0 x 10^6 cells/cm^2) in 6- or 96-well microplates, in tissue inserts (Falcon, Franklin Lakes, USA; or Anocell, Nunc, Roskilde, Denmark) or on glass coverslips (Kinitell). The electrophysiological and ultrastructural analyses were done in tissue inserts incorporating a polyethylene terephthalate (PT) membrane (surface area of 0.78 cm^2) (Falcon) with a collagen surface. Monolayers were used on the third day after seeding.

Murine skeletal muscle C2C12 was obtained from the American Type Culture Collection (CRL-1772, ATCC). Cytolysis was assessed in both undifferentiated myoblasts and differentiated myotubes derived from this cell line. Myoblasts were grown in 25 cm^2 flasks using Dulbecco's Modified Eagle's Medium (DMEM, Sigma), supplemented with 15% fetal calf serum (FCS, Sigma), 2 mM glutamine, 1 mM pyruvic acid, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 mg/ml), in a humified atmosphere with 7% CO_2 at 37 ºC. For experimental purposes, cells were harvested from subconfluent monolayers after exposure to trypsin (1500 U/ml) containing 5.3 mM EDTA for 5 min at 37 ºC. The resuspended cells were seeded in 96-well microplates, at an approximate initial density of 1–4 x 10^4 cells per well, in the same growth medium. After almost reaching the confluence, undifferentiated myoblasts were utilized directly in the cytotoxicity assay described below. In order to differentiate the cells to myotubes, the growth medium from the mioblasts grown in 96-well plate was aspirated and replaced by medium containing 1% FCS (Ebisui et al., 1995). After 3 to 5 additional days of culture, when a large proportion of long multinucleated myotubes was

observed among the myoblasts, cells were utilized in the cytotoxicity assay, as described below.

2.4. Cell viability

The cytotoxicity of the venom and PLA_2 (LmTX-I) in MDCK cells was tested using the neutral red dye assay. MDCK cells were seeded in 96-well microplates at a cell density of 1.96 x 10^5 cells/well. Following exposure to venom (*n* = 9 wells/group) or to the LmTX-I (*n* = 9 wells/group), the medium was removed and the cells were washed once with phosphate-buffered saline (PBS), pH 7.4. Two hundred microliters of MEM containing 50 mg of neutral red/ml was added to the cells and the plate was then incubated for 3 h at 37ºC. The medium containing the dye was removed and each well was washed once for 2-3 min with formol-calcium (40% formaldehyde and 10%, w/w, anhydrous calcium chloride) to remove non-incorporated dye. Finally, a mixture of acetic acid/50% ethanol (1:100, v/v) was added to each well for 15 min at room temperature, after which the plate was read at 540 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was determined by comparing the resulting absorbances with the mean absorbance of the control wells (without venom, considered as 100% viability).

Cytotoxic activity of the LmTX-I was assayed also in murine skeletal muscle C2C12 myoblasts and myotubes as described previously (Lomonte et al., 1999). Variable amounts of toxin were diluted in assay medium (DMEM supplemented with 1% fetal calf serum) to a final volume of 150 µl and added to cells in 96-well microplates. Reference controls for 0 and 100% toxicity were exposed to assay medium alone or with 0.1% Triton X-100, respectively. After 3 h at 37ºC, a supernatant aliquot was collected for

determination of lactic dehydrogenase (LDH; EC 1.1.1.27) activity released from damaged cells, using a colorimetric end-point assay (Sigma Nº500). Experiments were carried out in triplicate.

2.5. Measurement of transepithelial electrical resistance (R_T)

The transepithelial electrical resistance was measured across all MDCK monolayers using two Ag/AgCl "chopstick" electrodes coupled to a combined voltmeter and constant current source (EVOM, World Precision Instruments, UK).

2.6. Light microscopy

MDCK cells grown on coverslips (0.5 x 10^6 cell/coverslip) were incubated with *L. m. muta* venom (10 and 100 □g/ml) or LmTX-1 (100 □g/ml) at varying periods of time and then were fixed in ethanol: acetic acid (3:1, v/v) and stained by the Feulgen's reaction. Briefly, the cells were lysed in 4 N HCl for 75 min, washed with water, then treated with Schiff's reagent for 40 min, and finally washed with sulfuric water (0.5% sodium metabisulfite in 1 ml of 1 M HCl and 99 ml of water). The coverslips were subsequently mounted on glass slides.

2.7. Cytochemistry for F-actin in MDCK cells

MDCK cells grown on Anocell filters or coverslips (0.5 x 10^6 cell/filter or coverslip) were incubated with *L. m. muta* venom (10 and 100 □g/ml) or LmTX-1 (100 □g/ml) at varying periods of time. After time incubation, the monolayers were fixed in 2% paraformaldehyde (in 0.1 M phosphate-buffered saline, pH 7.4) for 10 min, permeabilized with 0.1% Triton-100X (in PBS) for 10 min and then incubated with

TRITC-conjugated phalloidin (1 □g/ml in PBS) in the dark for 1 h (Collares-Buzato *et al*., 1998). Afterwards the monolayers were washed several times with PBS and mounted with Vectashield (Vector Laboratories, Burlingame, USA) on glass slides. The stained cells were examined by confocal laser scanning microscopy (CLSM; Bio-Rad MRC 1024, Bio-Rad, Richmond, CA). To permit valid comparisons between the venom-treated/LmTX-1-treated and control groups, the labeling and microscopic examinations of all groups were done in the same experimental session.

2.8. Scanning Electron Microscopy (SEM)

For SEM, MDCK cells (0.5 x 10^6 cells/filter) grown on collagen-coated PT filters (Falcon) were fixed in 2.5% formaldehyde containing 0.06% picric acid, 1% tannic acid, 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) for at least 1h. After this time, the monolayers were washed with cacodylate buffer and then post-fixed in 2% aqueous solution of O_SO_4 at 4 ºC in the dark for 1 h. Monolayers were dehydrated through graded series of ethanol concentrations and then dried to the critical point using liquid CO_2 in a critical point dryer (Balzers CPD 030). Finally, they were mounted on metal stubs with double-sided stick tape and coated with gold using a sputter coater (Balzers SCD 050). Observations were made under a scanning electron microscope (MEV JEOL 5800 LV) at an accelerating voltage of 0.3–30 kV.

2.9. Statistical analysis

All numerical results were expressed, as the mean ± SEM. For multiple comparisons, the significance was assessed by ANOVA followed by the Tukey-Kramer multiple test. The level of significance was set at $p \leq 0.05$.

3. Results

The toxicity of *L. m. muta* venom was assessed by measuring neutral red cell uptake in MDCK epithelial cells. The amount of dye internalized by the population of cells in culture is directly proportional to the number of viable cells. At the highest concentration used (100 μg/ml), the venom significantly decreased the cell viability (to 70.2 ± 3.4 %) after 1 h incubation ($p < 0.05$) when compared to the control values (Fig. 1a). In addition, the venom at concentration of 10 μg/ml induced a decrease of approximately 25% in cell viability after 3 h incubation ($p < 0.05$). The decrease in cell viability induced by 100 μg venom/ml was time-dependent and after 5 h of incubation only 15% of the cells were viable. However, the venom at the concentration of 10 μg/ml induce a decrease in cell viability that did not show time dependence. Taken altogether, these data suggested a cytotoxic effect of the venom on cultured MDCK cells. Nevertheless, as shown in Fig. 1b, the LmTX-I at concentration of 100 μg/ml had no significant effect on cell viability of MDCK even after 24 h of incubation.

To ascertain whether the lack of cytotoxic effect shown by LmTX-I was not exclusively related to the type of cell line used (MDCK), we have tested the LmTX-I in another *in vitro* cell culture model, the skeletal muscle (C2C12) cell line. Established cell lines of skeletal muscle myoblasts have extensively used in this type of studies owing to their origin and to their relative susceptibility to myotoxins (Lomonte et al., 1994a). The release of lactic dehydrogenase (LDH) was quantified as a measure of membrane and

cell damage. As shown in Figure 2, the PLA_2 LmTX-I did not induce significant LDH release even 3 h after exposure of cells to concentrations of up to 270 μg of LmTX-I/ml (40 μg/well).

To investigate whether the venom and LmTX-I alter the epithelial barrier function (Collares-Buzato et al., 1994; 2002), the transepithelial electrical resistance (R_T) was measured across MDCK cells grown on permeable filters exposed to venom or to LmTX-I for 1, 3, 5 or 24 h. The initial R_T mean values of all experimental groups were similar. As shown in Figure 3, the crude venom (100 μg/ml) induced a significant decreased in R_T across MDCK monolayers after 1h of venom exposure (control R_T 1 h = 103.6 ± 10 % *vs* 100 μg venom/ml treated R_T 1 h = 67.5 ± 2.4 % (*n* = 5 monolayers)). We also detected significant alteration in this biophysical parameter following 3 h exposure to 10 μg of crude venom/ml or to 100 μg of LmTX-I/ml. However, a more prolonged exposure (up to 24 h) did not result in a further decrease in R_T across these monolayers treated with crude venom (10 μg/ml) or LmTX-I (100 μg/ml).

To assess alterations to the nuclear morphology following the treatments, MDCK cells grown on glass coverslips were stained with Feulgen's reaction after incubation with *L. m. muta* crude venom or LmTX-I (Fig. 4). The nuclear alterations induced by the treatment with 100 μg of crude venom/ml for 2 h included: reduction in the size of nuclei that often displayed varying degree of chromatin condensation (pycnosis) and apparent reduction in the number of mitotic nuclei (Fig. 4 b). Prolonged exposure to the venom at a concentration of 10 μg/ml, besides these alterations described above, resulted also in nuclear fragmentation of few cells (Fig. 4d). All these nuclear alterations are indicative of different stages of cell death (Clarke 1990). In addition, following venom incubation, cell

nuclei appeared more sparsely distributed throughout the MDCK monolayer as compared to the control group that may be result of cell detachment from the substratum (compare control in **a** *vs* treated in **b**). During the monolayer processing, we often observed cells detaching from the coverslip; the detachment was partial or total depending on the venom concentration and the time of incubation. Total monolayer detachment was seen with the highest concentration tested following 3 h of incubation, which prevent us to use these monolayers for morphological analysis. In contrast to that observed with crude venom, the monolayer exposure to LmTX-I (100 μg/ml) up to 5 h showed no significant changes in nuclear appearance or cell adhesion (Fig. 4 f).

Cellular morphology of MDCK cells following the treatments was assessed by SEM (Figure 5). Monolayer exposure to crude venom (10 μg/ml) for 5 h (Fig. 5b) and 24 h (Fig. 5e) resulted in morphological changes such as the presence of groups of cells with a fusiform (e) or a rounded (b) format that contrast with a more uniform and polygonal shape displayed by the control cells (Fig. 5a and d). In addition, treated monolayer showed areas of discontinuity due to cell detachment from the substrate (data not shown). Similar morphological alterations were seen after crude venom treatment for just 1 h with the highest concentration tested (100 μg/ml) (data not shown). In accordance with the results obtained by light microscopy, no alteration of cell morphology was observed even after 24 h of exposition to the LmTX-I (100 μg/ml) (Fig. 5c and f).

Since cell morphology depends directly on the cytoskeleton organization, we investigated the F-actin distribution using TRITC-conjugated phalloidin staining in MDCK cells following the experimental conditions (Figure 6). As depicted in Figure 6 a-c, control

cells displayed a punctuate staining of the apical cell surface (corresponding to the microvilli)(**a**), an intense staining of the lateral cell border at the area of cell-cell contact (corresponding to the perijunctional actin ring)(**b**) and staining of the stress fibers at the basal cell surface (**c**). No difference in this staining pattern was seen between control cells incubated for 2 h or for 5 h with culture medium. Nevertheless, MDCK cells when 5 h-exposed to 10 μg of *L. m. muta* venom/ml displayed a more fusiform or even an irregular format that contrasted with the polygonal normal shape of the control cells. In addition, the venom-treated (10 μg/ml) monolayer showed a shortening and a marked disarray of the stress fibers at the basal surface. Similar changes were observed after 2 h exposure to 100 μg/ml of *L. m. muta* venom (Fig. 6 g-i) except that, at this venom concentration, the treated cells seemed larger and some displayed a more rounded shape in comparison with the control. In addition, 100 μg venom/ml treated monolayer presented cells displaying discontinuities in the perijunctional actin ring (Fig. 6h). In contrast, TRITC-phalloidin staining revealed no marked changes in the distribution of F-actin filaments in MDCK cells following LmTX-I (100 μg/ml) exposure to 5 h (Fig. 6 j-l) in comparison to the control (Fig. 6a-c).

4. Discussion

This study showed that *L. m. muta* crude venom exerts toxic effects in cultured MDCK cells since we observed a significant decrease in neutral red cell uptake, a reduction in transepithelial electrical resistance, induction of nuclear alterations indicative of cell death, reduction in cellular adhesion to the substrate, and changes in cell morphology. In contrast, the LmTX-I, a basic phospholipase A_2 isolated from this

venom, displayed no significant cytotoxicity in both MDCK cells and in a skeletal muscle (C2C12) cell line even at the highest concentration tested (270 μg/ml).

The reduction in cell uptake of neutral red dye combined with the observed nuclear morphological changes indicates that the *L. m. muta* crude venom decreases cell viability in MDCK cells. The nuclear alterations following venom included chromatin condensation (pycnosis), reduction in the number of mitotic nuclei, and nuclear fragmentation that are indicative of varying stages of cell death (Clarke 1990). Nevertheless, at the moment we cannot tell whether the cell death is result of apoptosis or necrosis events triggered by the venom.

In addition, the *L. m. muta* venom induced a significant decrease in transepithelial electrical resistance across MDCK monolayers. The measurement of the transepithelial electrical resistance gives general information about the epithelial barrier function and integrity (Watlington et al. 1970; Kotyk and Janácek, 1977; Powell, 1981; Lo et al. 1999). So, changes in this biophysical measurement indicate that at least one of the following parameters of the cell monolayer has been altered: cell viability, cell membrane permeability, tight junction permeability and/or cell adhesion to the substrate. Since we demonstrated that the *L. m. muta* venom affects significantly both cell viability and cell adhesion, it is plausible to suggest that changes in these two parameters are the major contributors to the decrease in transepithelial electrical resistance observed across venom- treated MDCK monolayers. The observation that the crude venom impairs cell-to-substratum adhesion was further strengthened by the data showing that treated cells display a major disorganization of the stress fibers. In addition, the morphological alterations observed after treatment with the crude venom such as acquisition of a

fusiform shape and a larger size take us to believe that an attempt of reorganization of the cells is happening probably in order to restablish the integrity of the monolayer partially lost due to the cell detachment and cell death.

Similar alterations to the cell viability and cell-to-matrix adhesion have been observed with the venom of *Bothrops moojeni* (Collares-Buzato et al. 2002) and *B. alternatus* (Nascimento et al. 2004) using the same *in vitro* system. However, the venom from these different snake species seems to show differences in potency on MDCK cells. In comparison with *L. m. muta* venom, *Bothrops moojeni* and *B. alternatus* venom showed a more pronounced effect on neutral red cell uptake and transepithelial electrical resistance in MDCK cells. Incubation of MDCK cells for 3 h with 10 μ*g B. alternatus* venom/ml resulted in approximately 65% decrease in neutral red cell uptake (Nascimento et al. 2004) whereas the *L. m. muta* venom at the same concentration and time of incubation induced only 25% decrease in this parameter. Meanwhile, 5 h exposure to *B. moojeni* crude venom at concentration of 6.9 μg/ml induced a 78% decrease in R_T across MDCK monolayers that contrasted with a only 32% decrease in this measurement observed following 5 h incubation with 10 μg *Lachesis* venom/ml. The local symptoms of the envenoming by *Bothrops* and *Lachesis* genera are similar, that include edema, pain, hemorrhage, swelling, blistering, and necrosis, however, they differ from each other regarding the main systemic effects. With *Bothrops* venom, the primary cause of death is acute renal failure (ARF) followed by acute tubular necrosis and, occasionally, glomerulonephritis (Amaral et al., 1985; Burdmann et al., 1993). However, with *L. m. muta* venom, symptoms like vomiting, profuse diarrhea, hypotension, bradycardia are distinctive systemic symptoms (Jorge et al., 1997) and in some cases

the patient also show neurotoxic signs, like divergent strabismus, dysarthria and dysphagia (Torres et al., 1995). There are relatively few reports on renal complications after *L. m. muta* envenoming (Otero et al., 1992). Taken altogether, it seems that the *in vitro* effects of these different venoms in MDCK cells, a kidney-derived cell line, correlate well with the degree of renal toxicity observed following envenoming with these snake genera. This data envisages a possible use of the MDCK cell line as a good *in vitro* model for studying the intracellular mechanisms of action of venom that displays recognized kidney toxicity *in vivo*.

Lachesis m. muta venom is a mixture of a variety of proteins and enzymes; several of them have been isolated and characterized, including a L-amino acid oxidase (Sanchez and Magalhães, 1991), a lectin-like protein (Aragon-Ortiz et al., 1989); phospholipases A_2 (Fuly et al., 1997, 2002; Damico et al., 2005b); thrombin-like serine proteases (Torres et al., 1995; Magalhaes et al., 2003); metalloproteinases/desintegrins (Sanchez et al., 1987; Sanchez et al., 1995; Sanchez et al., 1995b; Rucavado et al., 1999); a proteinase with plasminogen-activating activity (Sanchez et al., 2000) and a kininogenase activity (Diniz and Oliveira, 1992; Giovanni-De-Simone et al., 1997). Some of these venom components can potentially contribute to the toxic effect seen in our *in vitro* system. Metalloproteinases/desintegrins are abundant in crotaline and viperine snake venoms and are known to contribute to the local myonecrosis and local/systemic hemorrhage seen *in vivo*, in part, due to their ability to degrade extracellular matrix and interfere with the cell-to-matrix adhesion (Ohsaka, 1979; Bjarnason and Fox, 1994; Kamiguti et al. 1998; Gutiérrez and Rucavado, 2000; Gallagher et al. 2003). LHF-II, one of the hemorrhagic metalloproteinases isolated from *Lachesis* venom, do not cause cell death but induce degradation of laminin, fibronectin and type IV collagen in culture of

capillary endothelial cells (Rucavado et al. 1999). So, we can suggest that metalloproteinases/desintegrins of the *L. m. muta* venom are the main responsible for the detachment of MDCK cells from the substrate following crude venom incubation as documented here.

Incubation of MDCK cells with crude venom resulted in cell death as demonstrated by the reduction in neutral red cell uptake combined with nuclear alterations as seen by microscopy. Putative candidates for the direct cytotoxicity of the venom may be the L-amino oxidases (LAAOs) and phospholipases A_2. The cytotoxic effects of LAAOs from snake venoms have been demonstrated in several cell lines (Ahn, et al., 1997; Masuda et al., 1997; Suzuki, et al., 1997). Most of the biological effects of LAAOs are believed to be result from a secondary effect of H_2O_2, which is produced in an enzymatic reaction (Li et al., 1994; Masuda et al., 1997; Torii et al., 1997). An increase in H_2O_2 concentration can induce membrane rupture, cell growth arrest and apoptotic cell death (Wiese et al., 1995; Suzuki et al., 1997; Toyama et al. 2006).

Regarding the PLA_2s, these enzymes are among the main factors responsible for the drastic necrosis of skeletal muscle observed in victims of envenoming by snakes (Mebs and Ownby, 1990; Gutiérrez and Lomonte, 1997; Harris, 2003). Some myotoxins of the group II PLA_2s cause a rapid cytolytic effect upon cell lines of skeletal muscle origin, as well as upon a variety of other cell types *in vitro* (Krizaj et al., 1991; Brusés et al., 1993; Bultrón et al., 1993; Bieber et al., 1994; Lomonte et al., 1994a, b; Incerpi et al., 1995). Four phospholipases A_2 have been identified and purified from *L. m. muta* venom: two basic PLA_2 isoforms, named LmTX-I and LmTX-II (Damico et al., 2005b) and two acidic isoforms, known as LM-PLA_2 and LM-PLA_2-II (Fuly et al. 1997, 2002). LmTX-I and LM-PLA_2 have been shown to display myotoxic activities in isolated muscle

preparations (Fuly et al., 2000; Damico et al., 2006). We showed herein that the LmTX-I had no effect on cell viability of MDCK cells and induced no significant changes in the cell release of lactic dehydrogenase in C2C12 myoblasts or myotubes. In agreement with this result, Gowda and Middlebrook (1993) reported the lack of cytotoxicity of two basic PLA_2 (group II) from *Vipera russelli* venom (named VRV-PL-V and VRV-PL-VIIIa) on skeletal muscle myoblasts. Therefore, according to our observations the LmTX-I, one of the constituents of the *L. m. muta* venom, probably do not contribute to the venom cytotoxicity in MDCK cells. Further work is needed to fully characterize the *L. m. muta* venom components in terms of their cytotoxic and myotoxic activities and, in our point of view, the cell culture constitutes a good tool to carry out these studies.

The LmTX-I did not alter either the nuclear and cellular morphology or the cellular distribution of F-actin filaments, revealed by TRITC-phalloidin staining, in MDCK cells. Nevertheless, 3 h incubation with 100 μg of LmTX-I/ml induced a steady but significant decrease in R_T across MDCK monolayers. As commented previously, changes in this biophysical measurement mean that at least one of the following parameters of the cell monolayer has been altered: cell viability, cell membrane permeability, tight junction permeability and/or cell adhesion to the substrate (Watlington et al. 1970; Kotyk and Janácek, 1977; Powell 1981; Lo et al. 1999). Since our work showed that the LmTX-I induces no cell death or impairment of cell adhesion, it is possible that the decrease in R_T induced by this enzyme reflects alterations of the membrane and/or junction permeabilities. Interestingly, previous works have documented that PLA_2s can affect directly membrane permeability to water and ions (Alix and Woodbury, 1997; Leite et al.

2004) and indirectly the tight junction permeability (Sawai et al. 2002) in epithelia. Taken altogether, our data show that the LmTX-I is not cytotoxic but displays catalytic activity.

Kini and Evans (1989), in order to explain the susceptibility of a tissue to a particular PLA_2 enzyme, proposed the presence of specific "target sites" on the surface of target cells or tissue. These target sites are recognized by specific "pharmacological sites" on the PLA_2 molecule (Kini, 2003). Therefore, the pharmacological sites of PLA_2 enzymes determine the affinity between the PLA_2 and the target proteins. The identification of pharmacological PLA_2 sites and the target proteins will help (a) to understand the structure-function relationships of PLA_2 enzymes; (b) to develop strategies to neutralize the toxicity and pharmacological effects by targeting these sites; as well as (c) to develop prototypes of novel research tools and pharmaceutical drugs (Kini, 2003). In the subgroup of Lys49 PLA_2 myotoxins, the C-terminal region (cationic/hydrophobic segment, 115-129) has been identified as crucial for the myotoxic/cytolitic activity (Lomonte et al., 1994a; Núñez et al., 2001; Chioato and Ward, 2003; Lomonte et al., 2003). However, the determination of the primary structure of LmTX-I (Asp49) revealed the absence of this C-terminal region rich in lisine (Damico et al., 2005b) that could partially explain the lack of cytolitic activity seen with this enzyme.

In conclusion, *L. m. muta* venom exerts a toxic effect on cultured MDCK cells that involves cell detachment from the substrate, morphological and nuclear alterations and a reduction in cell viability, with a consequent decrease in transepithelial electrical resistance of monolayers. The PLA_2 LmTX-I probably does not contribute to the direct venom cytotoxicity; these effects are probably mediated by metalloproteinases/disintegrins and other components of the venom.

Acknowledgments

D. C. S. D. is a recipient from a Doctoral fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior). This work was also supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo). The authors thank Mr. Paulo Baldasso and Ms Marta Beatriz Leonardo for technical support.

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Figure Legends

Figure 1. Cell viability of MDCK cells following *L. m. muta* venom (a) and PLA_2 (b) assessed by neutral red cell uptake assay. The control group remained in culture medium for the same period of time as the treated cells. The mean of neutral red uptake of the control group was taken as 100% dye uptake value. *$p < 0.05$ compared to the control group.

Figure 2. Cytolitic activity of LmTX-I from *L. m. muta* venom on C2C12 myoblasts or myotubes. Cytolysis was estimated by the release of lactic dehydrogenase (LDH) into supernatants after 3 h. Each point represents mean ± SD of triplicate cultures.

Figure 3. Decrease in transepithelial electrical resistance (R_T) across MDCK monolayers following *L. m. muta* venom and PLA_2 (LmTX-I). The control group remained in culture media for the same time period. All data are expressed as mean ± SEM of 5 monolayers per group. *$p < 0.05$ in comparison to the initial R_T value (at 0 h).

Figure 4. Feulgen reaction to detect changes in nuclear morphology of MDCK monolayers following *L. m. muta* venom 100 μg/ml for 2 h (b) or 10 μg/ml for 5 h (d) and PLA_2 100 μg/ml for 24 h (f). The control group remained in culture media for 2 h (a), 5 h (c) or 24 h (e). Arrows indicate fragmented nuclei and arrowheads show mitotic nuclei. Bar corresponds to 10 μm.

Figure 5. Scanning electron microscopy images of cultured MDCK monolayers following *L. m. muta* venom (10 μg/ml) for 5 h **(**b) or 24 h (e) and PLA_2 for 5 h (c) or 24 h (f). The control group remained in culture media for 5 h (a) or 24 h (d). Bars correspond to 10 μm.

Figure 6. Distribution of F-actin in MDCK monolayers following *L. m. muta* venom treatment with 10 μg/ml for 5 h (d-f) and 100 μg/ml for 2 h (g-i) or LmTX-I 100 μg/ml for 5 h (j-l) revealed by TRITC-phalloidin staining. The control group remained in culture media for 2 h (a-c). Panels are X-Y confocal images representing optical sections taken at the apical, medium or basal portions of the monolayers, using the same level of CLSM sensitivity for both experimental groups. Bars correspond to 50 μm.

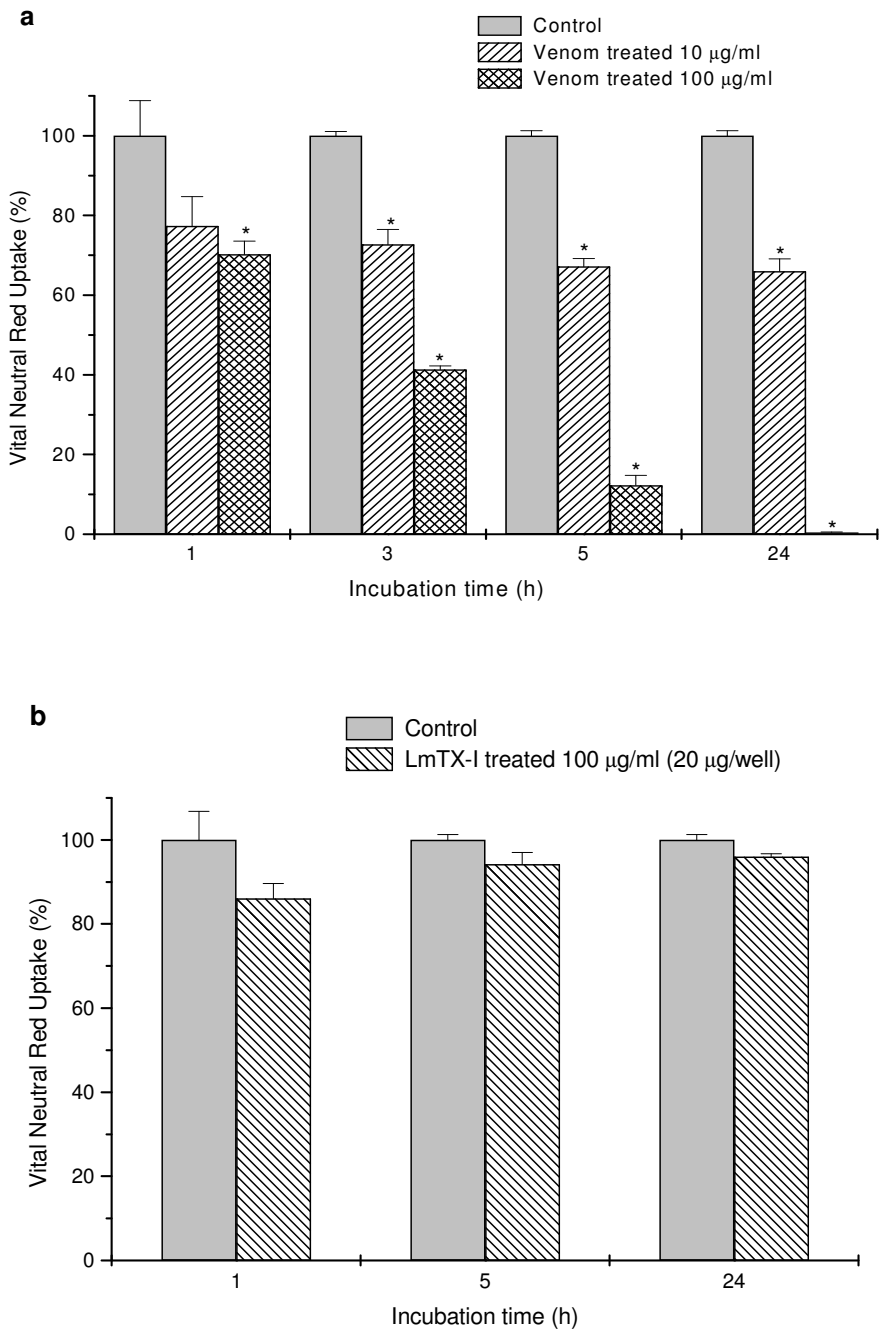
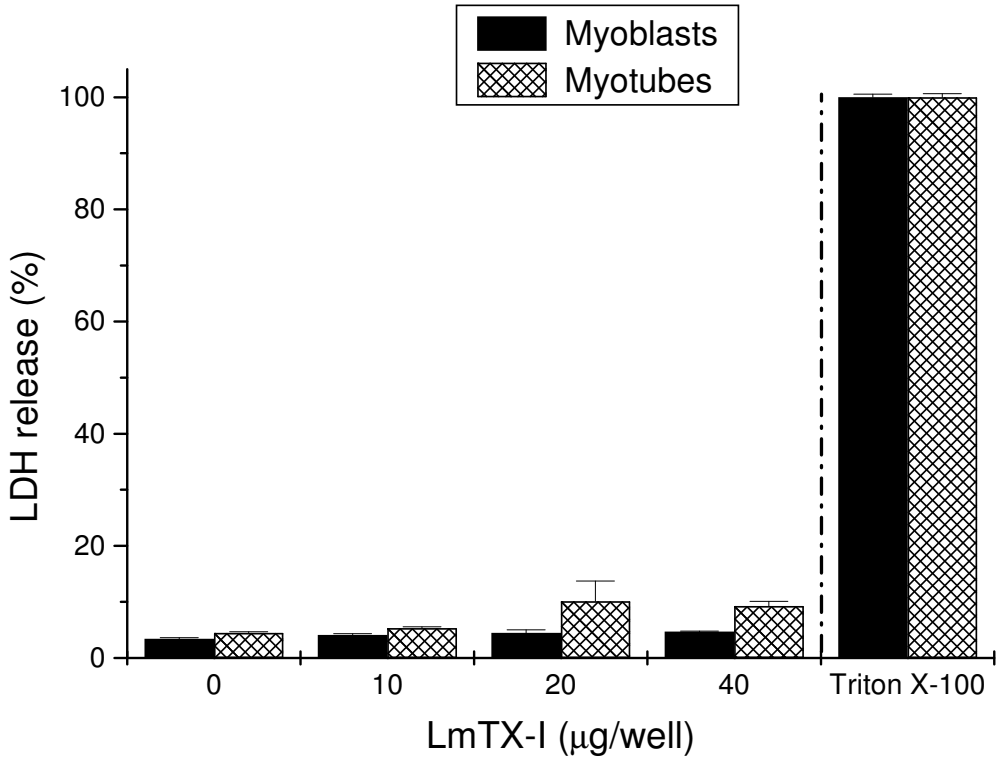


Fig. 1. Daniela C. S. Damico, Juliana M. do Nascimento, Luis A. Ponce-Soto, Bruno Lomonte, Paulo P. Joazeiro, Sérgio Marangoni, José Camillo Novello and Carla B. Collares-Buzato.



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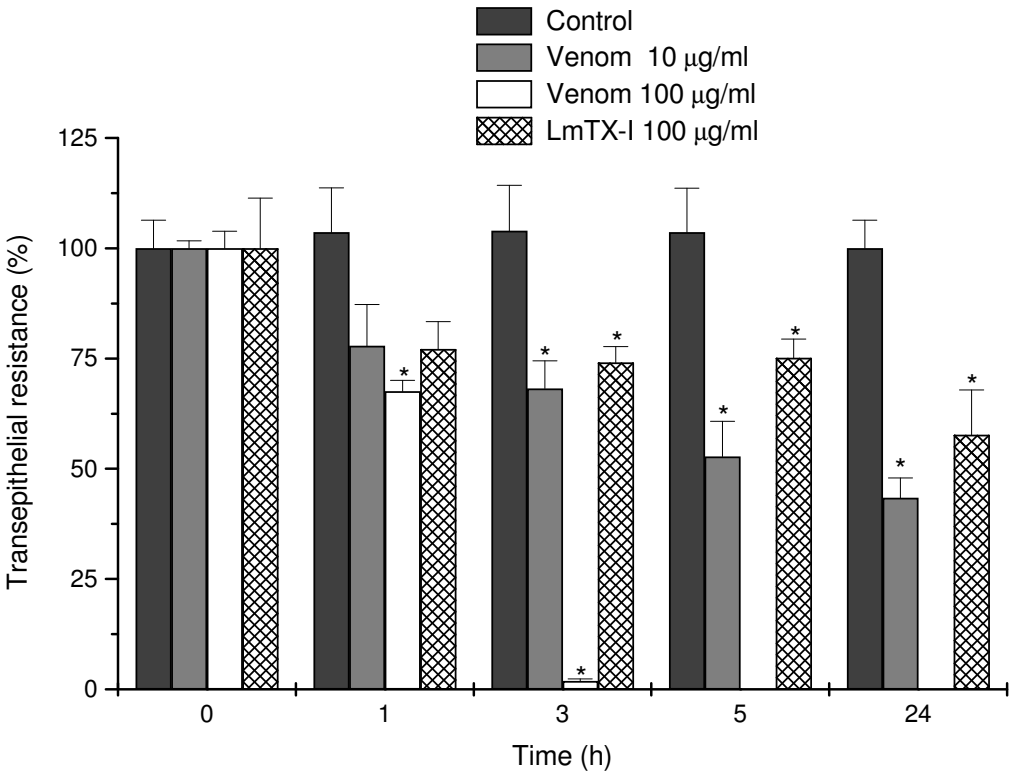

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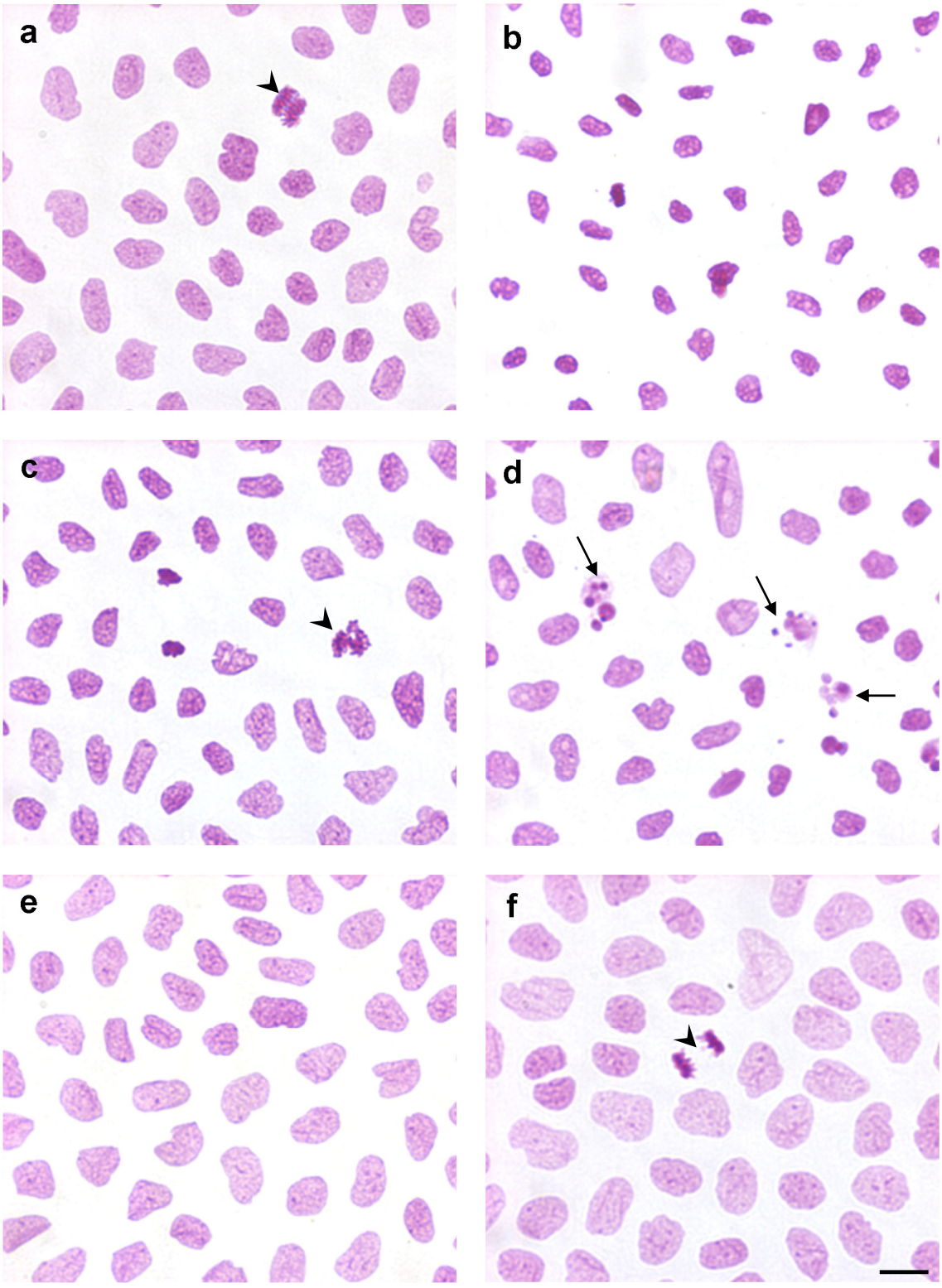

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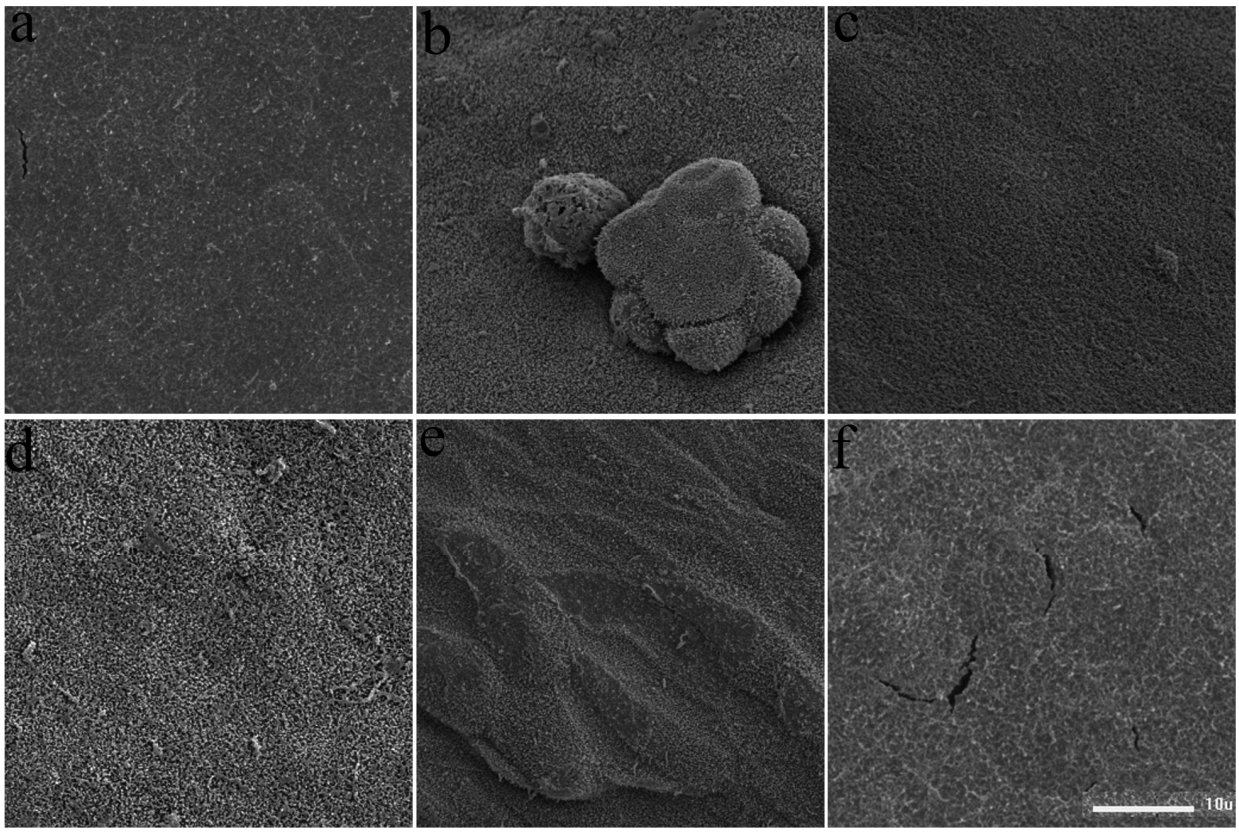

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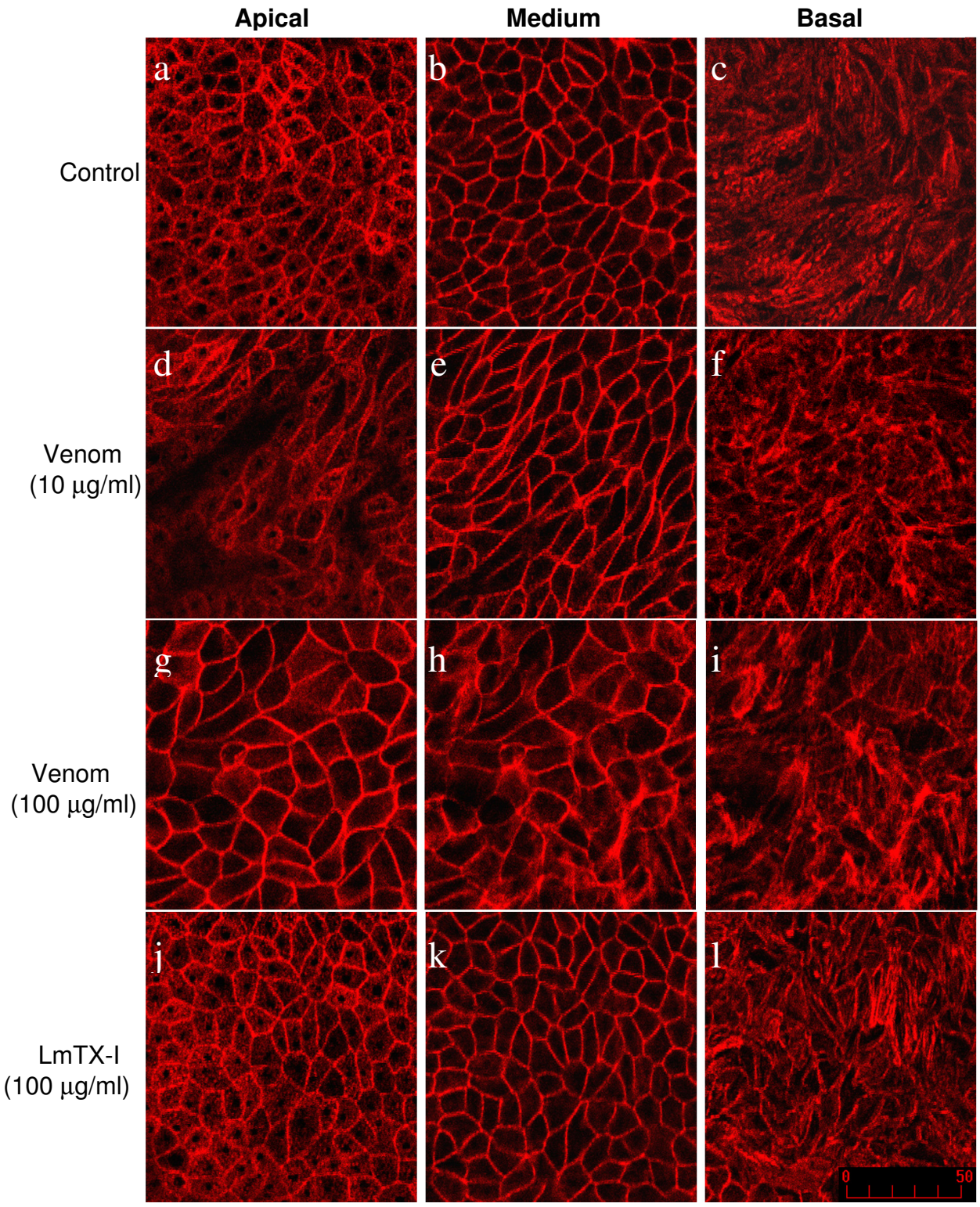

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CONCLUSÕES GERAIS

No capítulo 1 tivemos como conclusões principais:

- O veneno de *L. m. muta* possui uma ação neurotóxica bastante potente e uma ação miotóxica presente somente em concentrações mais altas (a partir de 10 μg/ml). Em concentrações tão baixas quanto 2 μg/ml de veneno na preparação biventer cervicis de pintainho foi visto bloqueio neuromuscular completo e irreversível, o que evidenciou a potente neurotoxicidade.

- Foi mostrado que a preparação de ave (músculo biventer cervicis) é mais susceptível ao veneno do que a preparação de mamífero (nervo frênico-diafragma de camundongo).

- Além disso, foi mostrado que este veneno possui uma ação predominantemente pré-sináptica, pois após o bloqueio neuromuscular total as preparações continuaram respondendo com contratura à adição de acetilcolina. Exceto na mais alta concentração usada (50 μg/ml) isto não foi observado. Por outro lado, o bloqueio da resposta ao potássio, com altas e médias concentrações da toxina (50 μg/ml e 10μg/ml), é um indício de que o veneno atua diretamente sobre a membrana muscular.

- A análise morfológica e o ensaio bioquímico de determinação da concentração de creatina quinase (CK) confirmaram que o veneno também é miotóxico, porém em concentrações mais altas do que aquelas em que foi observado a neurotoxicidade.

No capítulo 2:

- Foi isolado duas isoformas de PLA_2 Asp49 básicas, denominadas LmTX-I e LmTX-II, utilizando procedimentos relativamente rápidos e eficientes, baseado em cromatografia de exclusão molecular e HPLC de fase reversa. A massa molecular das duas isoformas, LmTX-I e LmTX-II, foi determinada como 14,245.4 e 14,186.2 Da, respectivamente.

- As duas proteínas foram seqüenciadas e diferenciada uma da outra pela substituição de um único resíduo de aminoácido, Arg65 (LmTX-I) $\rightarrow$ Pro65 (LmTX-II), sugerindo que provavelmente LmTX-I e LmTX-II sejam isoformas. A modificação deste resíduo não afetou a atividade catalítica de ambas as proteínas.

- A determinação da estrutura primária mostrou um alto grau de homologia entre as isoformas básicas de PLA_2 do veneno de *L. m. muta* e outras PLA_2 do veneno de outras serpentes pertencentes à família dos viperídeos (grupo II), como a mojave toxina, do veneno da *Crotalus s. scutulatus* e a crotoxina, do veneno da *Crotalus durissus terrificus*.

- LmTX-I apresentou atividade enzimática máxima entre 35 a 45 ºC e pH 8.0. Na presença de um substrato sintético, a PLA_2 mostrou um comportamento cinético do tipo

alostérico, visto especialmente com baixas concentrações do substrato. LmTX-I mostrou ser uma PLA_2 Ca^{2+} dependente, e sua atividade foi inibida por outros cátions .

No capítulo 3:

- A PLA_2 LmTX-I possui uma ação neurotóxica *in vitro* bastante potente (1 μg/ml), produzindo um bloqueio irreversível, concentração dependente, frente à preparação biventer cervicis de pintainho. Entretanto, com esta concentração, nenhum efeito miotóxico significativo foi produzido pela toxina. O efeito neurotóxico foi visto em concentrações bem menores que a necessária para se observar um efeito miotóxico significativo.

- Além disso, foi mostrado que LmTX-I possui uma ação preferencial em sítios pré-sinápticos, pois após o bloqueio neuromuscular total as preparações continuaram respondendo com contratura à adição da acetilcolina.

- A análise morfológica mostrou que as fibras musculares da preparação biventer cervicis de pintainho incubadas com a LmTX-I (30 μg/ml), sofreram alterações típicas de miotoxicidade. Este efeito miotóxico *in vitro*, foi confirmado pelo aumento na concentração de creatina quinase (CK) na solução do banho da preparação incubada com a toxina.

- Os resultados obtidos através da determinação da estrutura primária, aliados aos resultados biológicos, nos leva a concluir que a LmTX-I é uma PLA_2 Asp49 com atividade miotóxica e neurotoxicidade pré-sináptica, permitindo sugerir que a LmTX-I seja incluída em duas classes de toxinas: (1) no grupo II de PLA_2 de venenos, que agrupa aquelas encontradas em venenos de serpentes da família viperidae e (2) no grupo das β-neurotoxinas, que agrupa as PLA_2 neurotóxicas pré-sinápticas com atividade enzimática.

No capítulo 4:

- O estudo de citotoxicidade mostrou que o veneno total de *L. m. muta* exerce efeitos tóxicos em cultura de células MDCK, uma vez que, observamos uma significante diminuição na viabilidade celular, uma redução na resistência elétrica transepitelial através da monocamada, indução de alterações nucleares (indicativo de morte celular), redução na adesão celular ao substrato, e mudanças na morfologia celular.

- A PLA_2 (LmTX-I) não exerceu efeito citotóxico significativo (alteração na viabilidade celular, alterações nucleares ou morfológicas) perante os dois tipos celulares estudados (célula epitelial renal e célula muscular esquelética). Isso nos leva a acreditar, que os efeitos citotóxicos exercidos pelo veneno de *L. m.muta*, são provavelmente devido a outros componentes do veneno, como metaloproteinases/desintegrinas, L-amino oxidases, e até mesmo por outras isoformas de PLA_2 presentes neste veneno.

- Entretanto, a LmTX-I alterou a resistência elétrica transepitelial medida através da monocamada, possivelmente por alterar a permeabilidade da membrana.

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