ERICLEISON CARDOSO SILVA

CARACTERIZAÇÃO DE TOXINAS PRESENTES EM PEÇONHA DE VESPAS

Este exemplar corresponde à versão final da Dissertação de Mestrad apresentada à Pós-Graduação da Faculdade de Ciências Médicas UNICAMP, para obtenção do Título de Mestre em Farmacologia a Biólogo – Ericleison Cardoso Silva.

Campinas, 25 de fevereiro de 2003.

Prof. Dr. Stephen Hyslop
- Orientador -

CAMPINAS

2003

ERICLEISON CARDOSO SILVA

CARACTERIZAÇÃO DE TOXINAS PRESENTES EM PEÇONHA DE VESPAS

Dissertação de Mestrado apresentada ao Curso de Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Mestre em Farmacologia.

ORIENTADOR: PROF. DR. STEPHEN HYSLOP

CAMPINAS

2003

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS UNICAMP

Si38c

Silva, Ericleison Cardoso
Caracterização de toxinas presentes em peçonhas de vespas. / Ericleison Cardoso Silva. Campinas, SP : [s.n.], 2003.

Orientador : Stephen Hyslop
Dissertação (Mestrado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Vespas. 2. Peçonhas. 3. XXXXX. I. Stephen Hyslop II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

UNICAMP

Banca Examinadora da Dissertação de Mestrado

Orientador:

Prof. Dr. Stephen Hyslop

Membros:

Prof. Dr. Stephen Hyslop

Prof. Dr. Cleber Evandro Teixiera

Prof. Dr. Lanfranco Ranieri Paolo Troncone

Programa de Pós-Graduação em Farmacologia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 25/02/2003

A Deus que esteve presente em todos os meus momentos.

A minha esposa Cássia e aos meus pais, Erinaldo e Elindinalva, por todo apoio, dedicação e inesgotável amor.

AGRADECIMENTOS

Ao meu orientador Prof. Dr. Stephen Hyslop, pela dedicação dispensada em minha formação científica e pela amizade.

A Elza, Daiane, Sinval, Diego, Fábio Iara, Davi e toda minha família, que colaboraram com o apoio e incentivos para a realização desta etapa de minha vida.

Aos Professores Marco Aurélio Florêncio e Mônica Florêncio pelos incentivos, apoios e orientações em minha formação acadêmica e profissional, além da amizade e estima.

À Profa Dra. Léa Rodrigues-Simioni, pela orientação nos modelos experimentais de junções neuromusculares.

Aos Profs. Dr. Edson Antunes e Dr. Cléber Teixeira, pelos valiosos conselhos nos experimentos.

Às amigas de trabalho Pamela, Valéria, Alessandra, Alessandra Stroka, Christiane, Deise, Ágatha, Sueli, Marta e Patrícia, pelo companheirismo e amizade gratificantes.

Aos amigos Gildo Bernardo Leite e Cháristo André Dal Belo, pela colaboração, paciência e dedicação.

Ao técnico José Ilton dos Santos, pela dedicação no apoio técnico e amizade.

Ao Prof. Dr. José Carlos Freitas, pela calorosa recepção e orientação nos experimentos realizados em seu laboratório no CEBIMAR- USP.

Ao Prof Dr. Oton Meira Marques (Departamento de Zootecnia da Escola Politécnica de Agronomia – UFBA, BA), pela identificação taxonômica das vespas sociais.

Ao Prof. Dr. Sérvio Tùlio Pires do Amarante do Museo de Zoologia da USP (São Paulo), pela identificação das vespas solitárias.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo financiamento desta pesquisa.

A todos que colaboraram direta e indiretamente na realização deste trabalho.

Sumário

LISTA DE ABREVIATURAS

4-AP	4-Aminopiridina
5-HT	5-Hidroxitripitamina
ACh	Acetilcolina
ATP	Adenosina trifosfato
Bk	Bradicinina
EC_{50}	Concentração efetiva 50%
ED_{50}	Dose efetiva 50%
FLA_2	Fosfolipase A_2
Hist	Histamina
HPLC	Cromatografia líquida de alta performance
JNM	Junção neuromuscular
MCD	Degranulador de mastócitostions
MDTX	Mandaratoxina
MP	Mastoparano
MTX	Microbracontoxina
PbTx	Brevetoxina
TFA	Ácido trifluoroacético
TTX	Tetrodotoxina
α- e β-PMTX	α- e β-Pompilidotoxinas

LISTA DE FIGURAS

LISTA DE TABELAS

RESUMO

RESUMO

Peçonhas de vespas são capazes de produzir uma variedade de efeitos biológicos, tais como dor, edema, hipotensão, hepatotoxicidade e reações alérgicas. Com a exceção da peçonha da abelha *Apis mellifera* e de algumas (poucas) espécies de vespas sociais, as peçonhas de himenópteros brasileiros têm sido pouco estudadas. Neste trabalho, caracterizamos farmacologicamente as atividades nas peçonhas de três espécies de vespas sociais da família Vespidae (*Polybia chrysothorax*, *Pseudopolybia compressa* e *Polybia rejecta*) e duas espécies de hábitos solitários, da família Scoliidae, do gênero *Pypper (Pypper sp. 1 e Pypper sp. 2)*, todas da região nordeste do Brasil. Especificamente, avaliamos a atividade sobre músculo liso (íleo isolado de cobaia), a cardiotoxicidade em coração semi-isolado de barata, e a ação sobre neurotransmissão em nervo sensorial de crustáceo (siri azul, *Callinectes danae*) e em junção neuromuscular (preparação nervo frênico-diafragmo) de camundongo. As peçonhas das três espécies sociais contraíram o íleo isolado de cobaia na faixa de 0.001-100 μg/ml (dependendo da peçonha), com CE_{50} de 0,25 ± 0,05 μg/ml, 8,75 ± 1,25 μg/ml e 7,65 ± 1,85 μg/ml, (média±EPM, 1=4) para *Polybia chrysothorax*, *Polybia rejecta e Pseudopolybia compressa*, respectivamente. As peçonhas do gênero *Pypper* eram inativos neste tecido. A pré-incubação dos tecidos com atropina (antagonista dos receptores muscarínicos, 10 μM), metisergida (antagonista dos receptores 5-HT_2 da serotonina, 10 μM), e pirilamina (antagonista dos receptores H_1 da histamina, 10 μM) inibiu a atividade contrátil das três peçonhas sociais enquanto que o Hoe-140 (antagonista dos receptores B_2 da bradicinina, 1 μM) não afetou as respostas. Estes resultados indicam que as peçonhas sociais contêm aminas biogênicas (acetilcolina, histamina e serotonina) mas são destituídas de cininas. As três peçonhas (1-100 μg)

mostraram atividade cardiotóxica em coração semi-isolado de barata, sendo que as mais potentes foram *P. chrysothorax* e *P. compressa*; as peçonhas do gênero *Pypper* eram inativas nesta preparação. A investigação dos possíveis mecanismos responsáveis por este efeito através do uso de antagonistas/inibidores indicou que não houve envolvimento de aminas das peçonhas e que a causa mais provável era uma atuação a nível de canais iônicos, especialmente os de sódio e, em grau menor, os de potássio. A dosagem da atividade fosfolipásica das peçonhas mostrou a seguinte ordem de potência: *P. chrysothorax*/*P. compressa* > *P. rejecta* >> *Pypper* spp., porém esta atividade parece contribuir pouco para os efeitos observados uma vez que a indometacina apenas retardou o início da cardiotoxicidade. Na neurotransmissão, as peçonhas de *P. chrysothorax* e *P. compressa* (100 μg cada) aumentaram a atividade elétrica espontânea (disparo de potenciais de ação) do nervo sensorial de crustáceo de forma semelhante ao efeito causado pela brevetoxina (10^{-6} M), uma toxina marinha que age no mecanismo de portão do canal de sódio. As peçonhas também inibiram o potencial de ação do nervo em experimentos de "sucrose gap", efeito este que foi abolido pela tetrodotoxina (10^{-6} M), bloqueador de canais de sódio voltagem-dependentes. Nestes experimentos, as peçonhas de *P. rejecta* e do gênero *Pypper* eram inativas. As peçonhas das duas espécies sociais *P. chrysothorax* e *P. compressa* (25-100 μg/ml), mas não da social *P. rejecta* e das solitárias, inhibiram da neurotransmissão em preparações nervo frênico-diafragmo de camundongo (tempo para bloqueio de 50% da resposta contrátil: 40 ± 5 min (n= 3) e 57 ± 3 min (n= 3), respectivamente, na concentração de 100 μg/ml). Esta inibição era irreversível por lavagem das preparações. As peçonhas sociais também despolarizaram a membrana da fibra muscular e da placa terminal da junção neuromuscular. Estes achados sugerem uma ação

pós-sináptica com possível envolvimento pré-sináptico. As três peçonhas produziram alterações morfológicas no músculo do diafragma compatíveis com a mionecrose e correlacionada à sua atividade fosfolipásica. O fracionamento das peçonhas de *P. chrysothorax* e *P. compressa* por HPLC em coluna de fase reversa C_{18} resultou em vários picos. O principal destes da peçonha de *P. chrysothorax* foi seqüenciado. A seqüência obtida (ARSLLEGLGIRRGSA), indicou que o peptídeo pertence à família de peptídeos conhecidos como pompilitodotoxinas (PMTX), a qual contém duas isoformas, α-PMTX e β-PMTX, purificados das peçonhas das vespas solitárias *Anoplius samariensis* e *Batozonellus maculifrons*, respectivamente. Esta toxina reproduziu a cardiotoxicidade em coração de barata e o bloqueio de neurotransmissão em crustáceos observados para a peçonha de *P. chrysothorax*. Os resultados deste estudo indicam que as peçonhas investigadas possuem várias atividades biológicas. A demonstração de que as ações cardio- e neurotóxicas da peçonha de *P. chrysothorax* eram devidas em grande parte à presença de uma isoforma de PMTXs sugere que peçonhas de espécies de vespas sociais podem servir de fontes para moléculas com potencial utilidade como inseticidas.

INTRODUÇÃO

INTRODUÇÃO

Peçonhas de Himenoptera, particularmente das famílias Apidae (abelhas) e Vespidae (vespas), são uma rica fonte de peptídeos e proteínas com atividades biológicas. A peçonha de abelha é notável pelo conteúdo de melitina, apamina e peptídeo degranulador de mastócitos (mast cell degranulating peptide - MCD), mas não contém cininas, ao passo que estes últimos peptídeos são encontrados em muitas peçonhas de vespas e vespões que, por sua vez, não contêm melitina, apamina ou peptídeo MCD (HABERMANN, 1972; EDERY *et al.*, 1978; O'CONNOR e PECK, 1978). Além de peptídeos e proteínas, peçonhas de vespas também contêm substâncias de baixo peso molecular, tais como aminoácidos, aminas e catecolaminas (HABERMANN, 1972; EDERY *et al.*, 1978; NAKAJIMA, 1984; PIEK, 1991; GRIESBACKER *et al.*, 1998) (Tabela 1).

1. Substâncias de baixo peso molecular

1.1. Acetilcolina

A presença de acetilcolina tem sido relatada em peçonhas de vespídeos do gênero *Vespa crabro* (HABERMANN, 1972) e *V. orientalis* (EDERY *et al.*, 1972). A acetilcolina encontrada na peçonha destes vespídeos é responsável pela maioria dos efeitos provocados em alguns sistemas biológicos testados (EDERY *et al.*, 1978).

1.2 Aminas biogênicas

1.2.1 Histamina

A histamina é uma amina tecidual e está presente como um componente livre da peçonha de abelhas, vespas e serpentes (HABERMANN, 1972; EDREY *et al.*, 1978). Em humanos, a histamina da peçonha vespídeos contribue para a reação de dor e vasodilatação local que acompanha a picada (EDERY *et al.*, 1978).

1.2.2 5-Hidroxitriptamina (5-HT)

A 5-HT ou serotonina ocorre em grande variedade de peçonhas de artrópodes, sendo encontrada também no conjunto do aparato de ferrão de várias espécies de vespas (HABERMANN, 1972, EDERY *et al.*, 1978). A 5-HT promove a penetração do veneno na célula por aumento da permeabilidade celular (HABERMANN, 1972). Por outro lado, tem sido postulado que a 5-HT, sendo um potente algógeno, estenderia a contribuição para a resposta global de dor elicitada pela peçonha. A este respeito deve ser lembrado que a 5-HT potencializa o efeito algogênico de outras substâncias produtoras de dor, além de estimular as terminações nociceptivas (RANG e DALE, 1993; GOODMAN *et al.*, 1996).

Tabela 1. Principais constituintes de várias peçonhas de abelha, vespa e vespão.

	Abelha	Vespa	Vespão (Vespidae)
Aminas biogênicas			
Acetilcolina			+
Dopamina	+	+	
Histamina	+	+	+
Noradrenalina	+	+	
Serotonina		+	
			+
Peptídeos			
Apamina	+		
Cininas	+		
Melitina	+		
MCD	+		
		+	+
Enzimas			
Fosfolipase A	+	+	+
Fosfolipase B	+	+	+
Hialuronidase	+	+	

Fonte: HABERMANN (1972). MCD – peptídeo degranulador de mastócito.

1.2.3 Dopamina, noradrenalina e adrenalina

A concentração de dopamina em peçonhas de vespas é maior quando comparada com a de outras catecolaminas. Por outro lado, os níveis de dopamina, noradrenalina e adrenalina variam consideravelmente dentre as diferentes espécies de vespas e abelhas. A dopamina acelera a freqüência cardíaca de artrópodes, aumentando a circulação da hemolinfa, facilitando uma rápida distribuição de outros componentes tóxicos da peçonha (HABERMANN, 1972). Em humanos, as catecolaminas podem ser responsáveis pelo "efeito empalidecedor" da pele após a picada (EDERY *et al.*, 1972), devido à vasoconstrição e redução de sangue no local. Um efeito adicional das catecolaminas de peçonha de vespídeos seria a sensibilização dos receptores nociceptivos para algógenos (HABERMANN, 1972).

1.3 Aminoácidos

Estudos comparativos já demonstraram a presença de amino ácidos neuroativos em peçonha de vespões (gênero *Vespa* e *Vespula*) (ABE *et al.*, 1989). Estas substâncias agem como neurotransmissores inibitórios, no caso dos ω-amino ácidos GABA, β-Ala, Tau e Gly (CURTIS e WALKINS, 1965) e excitatórios, no caso da Glu, neurotransmissor na junção neuromuscular de insetos e que está presente em grande quantidade em peçonha de *Vespa* e *Vespula* (USHERWOOD *et al.*, 1968; ABE *et al.*, 1983,, 1989). Quantidades grandes destes aminoácidos transmissores promovem paralisia em insetos injetados (ABE *et al.*, 1989).

1.4 Peptídeos

Alguns peptídeos bioativos, tais como as cininas, mastoparanos, neurotoxinas e peptídeos quimiotácticos têm sido isolados e caracterizados da peçonha de vespídeos (YOSHIDA *et al.*, 1976; HO *et al.*, 1998). Vários destes peptídeos têm papel importante na dor, inflamação e inchaço que freqüentemente ocorrem depois da picada.

1.4.1 Cininas

As cininas são encontradas em peçonhas de vários grupos de Himenoptera, incluindo vespas sociais (Vespidae), três famílias de vespas solitárias (Scoliidae, Tiphiidae, Mutiliidae) e formigas (Formicidae) (PIEK, 1991). As cininas de peçonhas de vespas variam de 9 a 18 aminoácidos e são análogas às cininas de mamíferos, contendo na sua estrutura básica quase a mesma seqüência de aminoácidos que a bradicinina de mamíferos. Algumas cininas de vespídeos contêm carboidratos, como no caso das cininas de *Paravespula maculifrons* (YOSHIDA *et al.*, 1976) (Tabela 2).

As propriedades farmacológicas das cininas de vespas são semelhantes às das cininas encontradas em mamíferos (HABERMANN, 1972; NAKAJIMA *et al.*, 1985). Estes peptídeos provavelmente são os responsáveis por boa parte da dor sentida após a picada. Em insetos, as cininas de peçonhas de vespas bloqueiam a neurotransmissão pré-sináptica no sistema nervoso, levando à paralisia (HORI *et al.*, 1977; PIEK, 1991). As cininas conhecidas como megascoliacininas (YASUHARA *et al.*, 1987), da peçonha da vespa *Megascolia flavifrons*, bloqueiam os receptores nicotínicos de acetilcolina (PIEK, 1991).

1.4.2 Mastoparanos

Os mastoporanos são tetradecapeptídeos catiônicos ricos em aminoácidos hidrofóbicos, tais como alanina, isoleucina e leucina (PIEK, 1984; NAKAJIMA *et al.*, 1985). A Tabela 3 mostra a seqüência primária de alguns mastoparanos. Em todos os casos, estes peptídeos tem lisina nas posições 4, 11 e 12 (CHUANG *et al.*, 1996).

As ações biológicas dos mastoparanos em vários tipos de células incluem a liberação de serotonina de plaquetas de coelho, a liberação de catecolaminas e adenilato da adrenal bovina, hemólise de células cromafins renais e liberação de histamina de mastócitos peritoneais de ratos (NAKAJIMA *et al.*, 1998). A ação dos mastoparanos parece envolver múltiplas vias de transdução de sinal através de uma ativação direta de proteínas G, aumentando a atividade da GTPase e levando a diversas conseqüências intracelulares (HIGASHIJIMA *et al.*, 1998). O mastoparano B isolado da peçonha da *Vespa basalis* causa profunda queda na pressão sangüínea e disfunção cardíaca após injeção intravenosa em ratos, o que sugere um possível envolvimento deste peptídeo nos distúrbios cardiovasculares observados com a peçonha (HO *et al.*, 1994). Os mastoparanos também possuem potente atividade hemolítica, a qual atua em sinergia com uma proteína letal com atividade fosfolipásica A_1 presente na peçonha da *V. basalis* (HO e KO, 1988), causando hemólise e hemoglobinuria antes da morte em ratos.

Tabela 2. Cininas de peçonhas de Hymenoptera: comparação com cininas de mamíferos.

		1 2 3 4 5 6 7 9
Bradicinina (BK)	Cininas de mamíferos	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Kalidina (Lys-BK)		Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Met-Lys-BK		Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Vespa analis		Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Val-Ile
Vespa mandarinia		Gly-Arg-Pro-**Hyp**-Gly-Phe-Ser-Pro-Phe-Arg-Ile-Asp
Vespa tropica		Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Val-Val
Vespa xanthoptera		Ala-Arg-Pro-**Hyp**-Gly-Phe-Ser-Pro-Phe-Arg-Ile-Val
Paravespula maculifrons		Carbo. Carbo. Thr-Ala-Thr-Thr-Arg-Arg-Arg-Gly- Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Paravespula lewisii		Thr-Ala-Thr-Thr-Lys-Arg-Arg-Gly- Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg NacGal Gal
Polistes fuscatus		
Polistes exclamans		pGlu-Thr-Asn-Lys-Lys-Lys-Leu-Arg-Gly- Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Polistes annularis		
Polistes rotlmeyi		Arg-Pro-Pro-Gly-Phe-**Thr**-Pro-Phe-Arg
		Ala-Arg-Arg-Pro-Pro-Gly-Phe-**Thr** -Pro-Phe-Arg
		Arg-Arg-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Polistes jadwigae		Arg-Arg-Arg-Pro-Pro-Gly-Phe-**Thr** -Pro-Phe-Arg
		Arg-Thr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Polistes chinensis		Ser-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Parapolybia indica		pGlu-Glx-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg -Lys
Megascolia flavifrons		Arg-Pro-Pro-Gly-Phe-**Thr** -Pro-Phe-Arg
		Arg-Pro-Pro-Gly-Phe-**Thr** -Pro-Phe-Arg-Lys-Ala
Colpa interrupta		Arg-Pro-Pro-Gly-Phe-**Thr** -Pro-Phe-Arg

Fonte: PIEK (1991). As sequências sublinhadas correspondem à bradicinina. Carbo. – carbohidrato. Em negrito, os aminácidos que diferem da bradicinina.

Tabela 3. Seqüência primária dos mastoparanos MP, MP-X e MP-B.

MP	Ile - Asn -Leu - Lys -Ala -Leu -Ala -Ala -Leu -Ala -Lys -Lys -Ile -Leu -NH_2
MP-X	Ile -Asn -**Trp** -Lys -**Gly** -**Ile** -Ala -Ala -**Met** -Ala -Lys -Lys -**Leu** -Leu -NH_2
MP-B	**Leu** - **Lys** -**Leu** -Lys -**Ser** -**Ile** -**Val** -**Ser** - **Trp** -Ala -Lys -Lys -**Val** -Leu -NH_2

Fonte: CHUANG *et al.* (1996). Em negrito, os aminoácidos que diferem do MP

1.4.3 Neurotoxinas

A atividade neurotóxica da peçonha de vespas é bastante conhecida e indica a presença de uma variedade de toxinas com ação no sistema nervoso, principalmente de insetos e crustáceos (PIEK e SIMON THOMAS, 1969; GAWADE, 1983; PIEK, 1984). A ação destas toxinas pode ser periférica (pré ou pós-sináptica) ou central. Até o momento, em somente um pequeno número destas espécies a peçonha tem sido estudada.

A peçonha do vespão gigante *Vespa mandarina*, agressivo inseto do leste e sul da Ásia, cuja picada pode levar à morte em homens e animais domésticos, contém uma neurotoxina, a mandaratoxina (MDTX; peso molecular, 20.000), com potente ação sobre a neurotransmissão periférica (ABE *et al.*, 1982). Em preparações neuromusculares de lagosta, a aplicação desta toxina em quantidades nanomolares reduz a corrente de sódio nas fibras nervosas pré-sinápticas e, conseqüentemente, abole o potencial de ação pós-sináptico, sem alterar o potencial de repouso da membrana pós-sináptica. A peçonha da vespa *Microbracon hebetor* também exerce ação pré-sináptica em insetos através das

toxinas A-MTX e B-MTX com pesos moleculares de 43.700 e 56.700, respectivamente (VISSER *et al.*, 1983).

Peçonha de vespas sociais do gênero *Vespa*, tais como a *V. insularis* (HORI *et al.*, 1977; KAWAI *et al.*, 1979) e *V. mandarina* (KAWAI *et al.*, 1980), contêm neurotoxinas com ação pós-sináptica que envolve a abertura de canais de cloreto. No caso da vespa solitária *Philanthus triangulum*, a ação pós-sináptica é exercida pelas poliaminas filantotoxinas (α, β e γ) que bloqueiam canais juncionais e extrajuncionais da fibra muscular, reduzindo sua freqüência de abertura (PIEK, 1982; KITS e PIEK, 1986); a δ-filantotoxina pode também exercer uma ação central (PIEK, 1984). Toxinas poliaminas formam uma classe de compostos de baixo peso molecular, isolado de peçonhas de aranhas e vespas e que são os antagonistas de não-seletivos de receptores ionotrópicos como os de glutamato e receptores nicotínicos de acetilcolina (ELDEFRAWI *et al.*, 1988; BLASCHKE *et al.*, 1993; MUELLER *et al.*, 1995; STRMGAARD *et al.*, 2001). A α-pompilidotoxina (α-PMTX), um tridecapeptídeo isolado da peçonha da vespa aranha *Anoplius samariensis* (espécie solitária), facilita a neurotransmissão em preparações de crustáceos através de uma ação pós-sináptica (KONNO *et al.*, 1997, 1998) e pré-sinaptica, inativando a corrente de sódio de axônio de artrópodes (KONNO *et al*,. 2000; SAHARA *et al.*, 2000). Toxina semelhante, denominada β-pompilidotoxina (β-PMTX), também foi encontrada na peçonha de outra vespa aranha, *Batozonellus maculiforns*, e difere da α-PMTX na posição 12 onde a lisina é substituída pela arginina (Tabela 4). Em preparações neuromusculares de lagosta, a β-PMTX atua de modo semelhante à α-PMTX, porém é mais potente (KONNO *et al.*, 1998).

Tabela 4. Seqüência primária das pompilidotoxinas (PMTX) α e β.

α-PMTX	Arg-Ile-Lys-Ile-Gly-Leu-Phe-Asp-Lys-Leu-Ser-Lys-Leu-NH_2
β-PMTX	Arg-Ile-Lys-Ile-Gly-Leu-Phe-Asp-Lys-Leu-Ser-**Arg**-Leu-NH_2

Fonte: Konno *et al.* (1998). Em negrito, o aminoácido que difere da α-PMTX.

1.4.4 Peptídeos quimiotácticos

Os peptídeos quimiotácticos são tridecapeptídeos com elevado conteúdo dos aminoácidos Gly, Ile, Leu e Phe. Na peçonha da vespa social *Vespa tropica,* estes moléculas correspondem ao segundo maior grupo peptídico e foram as primeiras moléculas quimiotácticas a serem caracterizadas estruturalmente em peçonhas de artrópodes (HO *et al.*, 1998). Na relação estrutura-atividade, estes peptídeos contém a seqüência tripeptídica FLP que é importante na interação entre o peptídeo e o receptor quimiotáctico de neutrófilos. Os peptídeos quimiotácticos possuem apenas 20% da atividade liberadora de histamina encontrada nos mastoparanos (NAKAJIMA *et al.*, 1985). Comparado com outros peptídeos, a distribuição de peptídeos quimiotácticos em peçonhas de vespas têm sido bem menos estudadas.

2 Substâncias de alto peso molecular

Peçonhas de himenópteros contêm, além de aminas e petídeos (ver acima), enzimas, dentre elas: fosfolipase A, fosfolipase B e hialuronidase (EDERY *et al.*, 1978; HABERMANN, 1972; HO e KO, 1988).

2.1 Hialuronidase

A hialuronidase é considerada como responsável pela difusão no sítio de inoculação da peçonha por meio da despolimerização do ácido hialurônico (ALLALOUF *et al.*, 1972; Edery *et al.*, 1978). A função deste componente da peçonha de vespas pode ser associado, juntamente com a fosfolipase e o antígeno 5, uma proteína um alergênio com importante a e comportamento imunológico encontrada em peçonhas de vespideos, às reações alérgicas e atividades anafiláticas que acometem algumas vítimas de picadas de vespas (HOFFMAN *et al.*, 1984, 1985; LITTER *et al.*, 1985; KING *et al.*, 1996; HENRIKSEN *et al.*, 2001; PIRPIGNANI *et al.*, 2002).

2.2 Fosfolipases

Peçonhas de vespas contêm FLA_1 (VARGAS-VILLARREAL *et al.*, 1991; KING *et al.*, 1994; HO e KO, 1988), FLA_2 (OLIVEIRA e PALMA, 1998) e FLB (KING *et al.*, 1984; TAKASAKI e FUKUMOTO, 1989), sendo que esas enzimas são importantes alérgenos da peçonha (LITTER *et al.*, 1985; HOFFMAN *et al.*, 1985; KING *et al.*, 1996). As fosfolipases A estão envolvidas na liberação de ácidos graxos (FLA_1) e ácido araquidônico (FLA_2) dos fosfolipídeos de membranas celulares. O ácido araquidônico por sua vez pode ser metabolizado para prostaglandinas, tromboxanos e leucotrienos, importantes mediadores da inflamação. A FLA é capaz de produzir uma série de efeitos farmacológicos, tais como contração de músculo liso, queda da pressão arterial, aumento da permeabilidade capilar e degranulação de mastócitos (HABERMANN, 1972). As FLA podem também interferir com a transmissão neuromuscular (EDERY *et al.*, 1972). A FLA_2 presente na peçonha do vespão *Vespa basalis* é uma proteína letal que hidrolisa

fosfolipídeos (fosfatidilcolina e liso-fosfatidilcolina) e exerce uma ação hemolítica em eritrócitos do sangue de ratos e cobaias (HO e KO, 1988).

3 Toxinas inseticidas

Conforme já indicado acima, peçonhas de vespas e vespões contêm uma variedade de neurotoxinas, muitas das quais são específicas para insetos. Um grande número de neurotoxinas inseto-seletivas também tem sido isoladas de peçonhas de escorpiões e aranhas. As propriedades inseticidas são atribuídas à afinidade específica e exclusiva da ligação com os tecidos (receptores) de insetos. Por exemplo, a peçonha da aranha viúva-negra da família Theridionae contém a α-latrotoxina que causa uma rápida liberação de neurotransmissres dos botões pré-sinapticos (LONGENECKER *et al.*, 1970), uma outra toxina de aranha, da família Agelenidae, ω-agatoxina age nos canais de Ca^{2+} (ADAMS *et al.*, 1990; MINTZ *et al.*, 1992) e também PhTx-4 da peçonha da aranha *Phoneutria nigriventer* que inibe os receptores de glutamato e age nos canais de sódio despolarizando a membrana muscular e o nervo motor de insetos e mamíferos (FIGUEIREDO *et al.*, 2001). As α-toxinas e β-toxinas são polipeptídeos neurotóxicos presentes em peçonhas de escorpiões dos gêneros *Androctonus, Buthus, Buthotus* e *Leiurus* que associam-se aos sítios 3 e 4, respectivamente, dos canais de sódio de nervos, músculos e tecidos gladulares de mamíferos e insetos (LI *et al.*, 2000; BEN KALIFA *et al.*, 1997; SAUTIÈRE *et al.*, 1998; PELHATE e ZLOTKIN, 1982). Para peçonha de vespas, além das fosfolipases (VARGAS-VILLAREAL *et al.*, 1991; HO e KO, 1988; OLIVEIRA e PALMA, 1998; COSTA e PALMA, 2000) que são capazes de produzir uma série de efeitos farmacológicos, estão

presentes cininas neurotóxicas em Polistes e Scoliides, as quais causam bloqueio pré-sinaptico da transmissão colinérgica no sistema nervoso de insetos (PIEK, 1991). Os peptídeos neurotóxicos α e β-pompilidotoxinas (α e β-PMTXs) isoladas das vespas solitárias *Anoplius samariensis* e *Batozonellus maculifrons*, respectivamente, apresentam como ação um atraso na inativação da corrente de sódio (KONNO *et al.*, 1997, 1998, 2000; SAHARA *et al.*, 2000).

Considerando que aranhas, escorpiões e vespas são predadores naturais de insetos, os estudos destas inseto-toxinas podem promover a descoberta de novas moléculas para o desenvolvimento de novos inseticidas e biopesticidas.

OBJETIVOS

OBJETIVOS

O presente estudo propôs:

1- Investigar as atividades cardiotóxicas e neurotóxicas das peçonhas de vespas neotropicais em preparações de artrópodes.

2- avaliar as atividades sobre músculo liso e junção neuromuscular de mamíferos, com o intuito de comparar as ações tóxicas e a caracterização dos componentes ativos presentes nestas peçonhas.

ARTIGOS PARA PUBLICAÇÃO

1

Smooth muscle-contracting activity of some vespid venoms from northeastern Brazil

SHORT COMMUNICATION

Smooth muscle-contracting activity of some vespid venoms from northeastern Brazil

Ericleison Cardoso-Silva[1], Marco Aurélio P. Florêncio[2], Mônica. S. Florêncio[3], and Stephen Hyslop[1]

[1]Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil

[2]Centro Federal de Educação e Tecnológica (CEFET), Recife, PE, Brasil.

and

[3]Departamento de Ciências Morfológicas, Instituto de Ciências Biológicas, Universidade de Pernambuco (UPE), Recife, PE, Brasil.

Running title: Smooth muscle activity of vespid venoms

Author for correspondence: Dr. Stephen Hyslop, Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), CP 6111, 13083-970, Campinas, SP, Brasil. Tel. 55-19-3788-9536, Fax: 55-19-3289-2968, E-mail: hyslop@fcm.unicamp.br

Abstract

Wasp venoms contain a variety of low molecular weight substances, including amino acids, amines and bioactive peptides, many of which contribute to the effects of envenomation. In this work, we examined the smooth muscle contracting activity of venom from three species of neotropical social wasps (*Polybia chrysothorax*, *Polybia rejecta* and *Pseudopolybia compressa*, family Vespidae) found in northeastern Brazil. All three venoms produced concentration-dependent contractions in guinea pig isolated ileum with EC_{50} of 0.25 ± 0.05, 8.75 ± 1.25 and 7.65 ± 1.85 µg/ml (mean±S.E.M., n=4) for *P. chrysothorax*, *P. rejecta* and *P. compressa*, respectively. The shapes of the dose-response curves for *P. chrysothorax* and *P. rejecta* were very similar, whereas that of *P. compressa* was less steep. Pretreating the tissues with atropine (muscarinic receptor antagonist), methysergide ($5\text{-}HT_2$ receptor antagonist) or pyrilamine (H_1 receptor antagonist) blocked the contractile activity of the venoms to varying degrees. In contrast, Hoe-140, a bradykinin B_2 receptor antagonist, had no effect on venom-induced contractions. These results indicate that the main smooth-muscle contracting substances of the venoms are acetylcholine, histamine and serotonin, with no contribution by kinins.

Key words: guinea pig ileum, histamine, kinins, serotonin, smooth muscle, venom, wasp.

1. Introduction

Hymenopteran (ant, bee and wasp) venoms contain a variety of low molecular weight substances, including amino acids (Abe et al., 1989), amines (principally histamine and serotonin) (Owen and Bridges, 1982; Piek et al., 1983a,b; Sarangi et al., 1990), and peptides (Piek et al., 1989; Piek, 1984, 1991; Sarangi et al., 1990; Ho et al., 1998; Konno et al., 2001, 2002), but are generally poor in enzymes, except for hyaluronidase and phospholipases (Edery et al., 1978; Nakajima, 1984; Piek, 1984; Schmidt et al., 1986; Ho and Ko, 1988). In addition to their presence in venoms, several endogenous mediators, especially amines, may be released by venom components following envenomation (Ho and Wang, 1991; Ho et al., 1993; Eno, 1997; Griesbacker et al., 1998).

Despite the numerous species of stinging hymenopterans in Brazil, very little is known of their toxinology. The best studied species is the honey bee *Apis mellifera* (Azevedo-Marques et al., 1992; Fan and França, 1992; Ferreira et al., 1995; dos Reis et al., 1998), mainly because of its clinical importance. However, in recent years, the venoms of several social wasp species, including *Agelaia pallipes pallipes* (Costa and Palma, 2000), *Polybia paulista* (Oliveira and Palma, 1998), *Polistes versicolor* (Vargas-Villarreal et al., 1991) and *Protonectarina sylveirae* (Dohtsu et al., 1993), have been characterized biochemically, with the isolation of phospholipases and peptides. In this work, we examined the smooth muscle contracting activity of the venom of three species of social wasps found in northeastern Brazil.

2. Material and methods

2.1. Reagents

Atropine, bradykinin, histamine, indomethacin, methysergide, pyrilamine and serotonin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The bradykinin B_2 receptor antagonist Hoe-140 was from Hoescht (Germany). Other reagents of analytical grade were obtained from J.T. Baker (Xalostoc, Mexico), Mallinckrodt (Paris, KY, USA) or Merck (Rio de Janeiro, RJ, Brazil).

2.2. Venom collection

Colonies of the social wasps *Polybia chrysothorax, Polybia rejecta* and *Pseudopolybia compressa* were collected in the coastal forest zone of the state of Pernambuco in northeastern Brazil. The insects were killed with CO_2 and stored to -20°C until used. When required, the wasps were thawed on ice and the venom sacs removed along with the sting. Following removal of the sting, the venom was obtained by gently pressing (without squashing) the venom sacs in a beaker to expel their liquid content after which the sacs were stirred gently in distilled water with a magnetic stirrer at 4° C to ensure maximum recovery of venom. The venom sacs were subsequently removed by centrifugation (4200 rpm, 10 min) and the supernatant then recovered, lyophilized and stored to -20° C until required. The protein content of the material thus obtained was determined by the method of Lowry as modified by Peterson (1977), using bovine serum albumin as a standard. All amounts of venom mentioned below refer to this protein content.

2.3. Guinea pig isolated ileum

The preparations were set up as described elsewhere (The Staff, 1968). Male Hartley-Duncan guinea pigs (700-900 g) were killed with a lethal dose (>50 mg/kg, i.p.) of sodium pentobarbital (Hypnol®, Cristália, Itapira, SP, Brazil) after which the ileum was removed and placed in Tyrode solution (composition, in mM: NaCl 137, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $CaCl_2$ 1.8, $MgCl_2$ 0.49, KCl 2.7 and glucose 5.55). Fragments of ileum 2-3 cm long were cleaned of mesenteric tissue and mounted under a tension of 2.5 g in a 10 ml organ bath. The Tyrode solution was oxygenated continuously with a mixture of 95% O_2/5%CO_2 at 37°C. After allowing 30 min for stabilization, concentration-response curves were obtained for acetylcholine, bradykinin, histamine, serotonin (5-hydroxytryptamine) and the venoms. Agonists and venoms were left in contact with the tissue until maximum responses had been achieved after which the preparations were washed with Tyrode solution before the next concentration or treatment. The involvement of various mediators in the venom-induced contractile responses was assessed by pre-incubating the preparations with atropine (muscarinic receptor antagonist; 10 μM), methysergide (serotonin 5-HT_2 receptor antagonist; 10 μM), Hoe-140 (bradykinin B_2 receptor antagonist; 1 μM) or pyrilamine (histamine H_1 receptor antagonist; 10 μM) for 5-30 min (The Staff, 1968). The muscle contractions were recorded via isotonic transducers (Havard Apparatus, Harvard, MA, USA) coupled through amplifiers to a Linearcorder Mark VII - WR3101 six-channel recorder (Graphtec Corp., Japan). The contractions were expressed as a percentage of the maximum tissue response obtained for each agonist or relative to the response obtained with a maximally effective concentration of acetylcoline (15 μM). Concentration-response curves were constructed and EC_{50} for the agonists and venoms were calculated using the software

package GrapPad Prism, v. 2.01 (GraphPad Software Incorporated., CA, USA). The results were expressed as the mean±S.E.M. and were compared using Student's *t*-test, with a value of $p<0.05$ being considered significant. The statistical calculations were done using Prism software.

3. Results and discussion

Figure 1 shows the dose-response curves for the venoms in guinea pig isolated ileum. The EC_{50} for the venoms were 0.25±0.05, 8.75±1.25 and 7.65±1.85 μg/ml for *P. chrysothorax*, *P. rejecta* and *P. compressa*, respectively. The curves for *P. chrysothorax* and *P. rejecta* were parallel to each other but that of *P. rejecta* was shifted to the right by ~35 fold. In contrast, the curve for *P. compressa* was less steep than for the other two venoms. Figure 2 shows concentration-response curves for acetylcholine, histamine, 5-hydroxytryptamine and bradykinin in the absence and presence of their respective receptor antagonists. The EC_{50} for all agonists increased significantly in the presence of antagonists, indicating that there was effective receptor blockade by the antagonists used. Similar curves for the venoms were not obtained because of the limited amount of material available. However, as shown in the right hand panels of Figure 2, atropine, methysergide and pyrilamine antagonists significantly reduced the responses to single concentrations of each venom. The blockade by atropine was greatest for *P. compressa* whereas methysergide and pyrilamine were most effective in *P. chryosothorax* and *P. rejecta*. These results indicate that acetylcholine, histamine and serotonin are involved to varying degrees in non-vascular smooth muscle contraction by these venoms. The greater inhibition by methysergide and pyrilamine of the contractions caused by the venoms of *P. chrysothorax* and *P. rejecta*

suggests that these venoms may contain more serotonin and histamine and agrees with their taxonomic relatedness (same genus). In contrast, *P. compressa*, which belongs to a different genus, apparently contained more acetylcholine than the other two venoms.

These findings generally agree with the presence of these substances in other wasp venoms. Thus, acetylcholine occurs in large amounts in the venoms of the social wasps *Vespa cincta* (Sarangi et al., 1990), *Vespa crabro* (Habermann, 1972) and *Vespa orientalis* (Edery et al., 1978) and the solitary wasp *Philanthus triangulum*, which contains no histamine or serotonin (Piek et al., 1983b). Histamine has been identified in venom of the wasps *Megascolia flavifrons* (Piek et al., 1983a), *Vespa cincta* (Sarangi et al., 1990), and *Vespa crabro* and *Vespa vulgaris* (Edery et al., 1978), and in a variety of solitary species (Piek et al., 1983b). Serotonin has been detected in the venoms of *V. orientalis, V. vulgaris* and *P. rejecta* (Edery et al., 1972, 1978) and in other vespids (Owens and Bridges, 1982).

In contrast to the presence of the above mediators, the bradykinin B_2 receptor antagonist Hoe-140 did not affect the venom-induced contractions (Fig. 2), indicating that kinins are not involved in the ileum responses to *P. chrysothorax, P. compressa* and *P. rejecta* venoms. This finding is interesting since kinins occur in the venoms of various Hymenoptera, particularly social wasps (Vespidae) and ants (Formicidae) (Piek et al., 1989; Piek, 1991). Kinins appear to be more common in social than in solitary wasp venoms (Piek et al., 1983b, 1989), and these peptides have been found in few species of the latter group of wasps (Konno et al., 2001, 2002). Wasp venom kinins consist of 9 to 18 amino acids and may contain carbohydrate, as in the case of *Paravespula maculifrons* (Yoshida et al., 1976). The pharmacological properties of wasp kinins are similar to those of mammalian kinins (Nakajima et al., 1985), and these peptides are at least partly responsible for the

increased vascular permeability and pain associated with envenomation. In insects, wasp kinins cause paralysis by blocking cholinergic neurotransmission via nicotinic receptors (Piek, 1991). The factors influencing the distribution of kinins in wasp venoms have not been determined but may well be related to the susceptibility of the insect prey of each wasp species.

In conclusion, the venoms of the three species investigated here contained primarily amines (acetycholine, histamine and serotonin), with no involvement of kinins. The amines may contribute to some of the local effects such as pain and erythema seen following envenomation.

Acknowledgments

The authors thank José Ilton dos Santos, Gildo Bernardo Leite and Emanuela Barreto de Sousa for technical help, Dr. Edson Antunes (Departamento de Farmacologia, UNICAMP, Campinas, Brazil) for advice and for providing some of the drugs used in this study and Dr. Oton Meira Marques (Escola Politécnica de Agronomia, UFBA, Salvador, Bahia, Brazil) for identifying the wasps. E.C.S. was supported by a studentship from FAPESP (grant no. 00/05976-6). S.H. is supported by a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Abe, T., Hariya, Y., Kawai, N., Niwa, A. 1989. Comparative study of amino acid composition in an extract from hornet venom sacs: high content of neuroactive amino acids in *Vespa*. Toxicon 27, 683-688.

Azevedo-Marques, M.M., Ferreira, D.B., Costa, R.S., 1992. Rhabdomyonecrosis experimentally induced in Wistar rats by Africanized bee venom. Toxicon 30, 344-348.

Costa, H., Palma, M.S. 2000. Agelotoxin: a phospholipase A_2 from the venom of the neotropical social wasp cassununga (*Agelaia pallipes pallipes*) (Hymenoptera – Vespidae). Toxicon 38, 1367-1379.

Dohtsu, K., Okumura, K., Hagiwara, K., Palma, M.S., Nakajima, T. 1993. Isolation and sequence analysis of peptides from the venom of *Protonectarina sylveirae* (Hymenoptera-Vespidae). Nat. Toxins 1, 271-276.

dos Reis, M.A., Costa, R.S., Coimbra, T.M., Teixeira, V.P. 1998. Acute renal failure in experimental envenomation with Africanized bee venom. Ren. Fail. 30, 39-51.

Edery, H., Ishay, J., Gitter, S. 1972. Pharmacological activity of oriental hornet (*Vespa orientalis*) venom. Toxicon 10, 13-23.

Edery, H., Ishay, J., Gitter, S., Joshua, H. 1978. Venoms of Vespidae. In: Bettini, S. (Ed.), Handbook of Experimental Pharmacology, Vol. 48. Arthropod Venoms. Spinger Verlag, Berlin, pp. 691-771.

Eno, A.E. 1997. Pharmacological investigation of oedema induced by venom from the wasp *Polistes fuscatus*. Toxicon 35, 1691-1698.

Fan, H.W., França, F.S.O. 1992. Acidentes por himenópteros. In: Schvartsman, S. (Ed.), Plantas Venenosas e Animais Peçonhentos. Editora Sarvier, São Paulo, pp. 228-232.

Ferreira, D.B., Costa, R.S., de Oliveira, J.A., Muccillo, G. 1995. An infarct-like myocardial lesion experimentally induced in Wistar rats with Africanized bee venom. J. Pathol. 177, 95-102.

Griesbacker, T., Althuber, P., Zenz, M., Rainer, I., Griengl, S., Lembeck, F. 1998. *Vespula vulgaris* venom: role of kinins and release of 5-hydroxytryptamine from skin mast cells. Eur. J. Pharmacol. 351, 95-104.

Habermann, E. 1972. Bee and wasp venoms. Science 177, 314-322.

Ho, C.L., Chen, W.C., Lin, Y.L. 1998. Structures and biological activities of new wasp peptides isolated from the black-bellied hornet (*Vespa basalis*) venom. Toxicon 36, 609-617.

Ho, C.L., Hwang, L.L. 1991. Local edema induced by the black-bellied hornet (*Vespa basalis*) venom and its components. Toxicon 29, 1033-1042.

Ho, C.L., Hwang, L.L., Chen, C.T. 1993. Edema-inducing activity of a lethal protein with phospholipase A_1 activitiy isolated from the black-bellied hornet (*Vespa basalis*) venom. Toxicon 31, 605-613.

Ho, C.L., Ko, J.L. 1988. Purification and characterization of a lethal protein with phospholipase A_1 activity from the hornet (*Vespa basalis)* venom. Biochim. Biophys. Acta 963, 414-422.

Konno, K., Hisada, M., Naoki, H., Itagaki, Y., Yasuhara, T., Juliano, M.A., Juliano, L., Palma, M.S., Yamane, T., Nakajima, T. 2001. Isolation and sequence determination of peptides in the venom of the spider wasp (*Cyphononyx dorsalis*) guided by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. Toxicon 39, 1257-1260.

Konno, K., Palma, M.S., Hitara, I.Y., Juliano, M.A., Juliano, L., Yasuhara, T. 2002. Identification of bradykinins in solitary wasp venoms. Toxicon 40, 309-312.

Nakajima, T. 1984. Biochemistry of vespid venom. In: Tu, A.T. (Ed.), Handbook of Natural Toxins, Vol. 2. Insect Poisons, Allergens and other Invertebrate Venoms. Marcel Dekker, New York, pp. 110-133.

Oliveira, M.R., Palma, M.S. 1998. Polybiatoxins: a group of phospholipases A_2 from the venom of the neotropical social wasp paulistinha (*Polybia paulista*). Toxicon 36, 189-199.

Owen, M.D., Bridges, A.R. 1982. Catecholamines in honey bee (*Apis mellifera* L.) and various vespid (Hymenoptera) venoms. Toxicon 20, 1075-1084.

Peterson GL (1977) A simplification of the protein assay methodof Lowriy *et al.* Which is more generally applicable. Annal Biochem. 83, 357-363.

Piek, T. 1984. Pharmacology of Hymenoptera venoms. In: Tu, A.T. (Ed.), Handbook of Natural Toxins, Vol. 2. Insect Poisons, Allergens and other Invertebrate Venoms. Marcel Dekker, New York, pp. 135-185.

Piek, T. 1991. Neurotoxic kinins from wasp and ant venoms. Toxicon 29, 139-149.

Piek, T., Buitenhuis, A., Simonthomas, R.T., Ufkes, J.G., Mantel, P. 1983a. Smooth muscle contracting compounds in the venom of *Megascolia flavifrons* (Hym: Scoliidae) with notes on the stinging behaviour. Comp. Biochem. Physiol. 75C, 145-152.

Piek, T., Buitenhuis, A., Veldsema-Currie, R.D., Mantel, P. 1983b. Smooth muscle contracting compounds in venom of sphecid wasps (Hym: Sphecidae). Comp. Biochem. Physiol. 75C, 153-159.

Piek, T., Schmidt, J.O., de Jong, J.M., Mantel, P. 1989. Kinins in ant venoms – a comparison with venoms of related hymenoptera. Comp. Biochem. Physiol. 92C, 1171-1124.

Sarangi, B., Kar, P.K., Datta, A., Gomes, A., Lahiri, S.C. 1990. Isolation, partial purification and pharmacodnynamics of a slow contractile substance in the venom sac extract of the wasp *Vespa cincta* Fabr. Indian J. Exp. Biol. 28, 557-561.

Schmidt, J.O., Blum, M.S., Overal, W.L. 1986. Comparative enzymology of venoms from stinging Hymenoptera. Toxicon 24, 907-921.

The Staff. 1968. Pharmacological Experiments on Isolated Preparations. E&S Livingstone, Edinburgh.

Vargas-Villarreal, J., Martin-Polo, J., Reynaud, E., Alagon, A.C. 1991. A new affinity adsorbent for the purification of phospholipases A_1 and A_2 from animal venoms. Toxicon 29, 119-124.

Yoshida, H., Geller, R.G., Pisano, J.J. 1976. Vespulakinin: new carbohydrate-containing bradykinin derivate. Biochemistry 15, 61-64.

FIGURE LEGENDS

Figure 1. Dose-response curves for social wasp venoms in guinea pig isolated ileum. The preparations were mounted as described in Methods. The contractions are expressed as a percentage of the maximum response produced by each agonist. The points are the mean±S.E.M. of four experiments.

Figure 2. Concentration-response curves for acetylcholine (ACh, A), histamine (Hist, B), serotonin (5-HT, C) and bradykinin (Bk, D) in guinea pig isolated ileum in the absence and presence of their respective receptor antagonists. The EC_{50} (in μM) for ACh, histamine, 5-HT and Bk were, respectively, 0.10±0.02, 0.05±0.04, 0.07±0.02, and 0.05±0.01 before and 3.3±0.1, 5.7±1.6, 1.02±0.08 and 0.4±0.07 after the corresponding antagonists ($p<0.05$ in all cases for before *vs.* after). The right hand panels show the effect of the various antagonists on venom-induced contractions. Note that because of the limited amount of venoms available, it was not possible to obtain full dose-response curves for these. The concentration of each venom tested was that required to produce approximately 75% of the maximum response for each venom. The points and bars are the mean±S.E.M. of 3-4 experiments. *$p<0.05$ compared to the responses before antagonist.

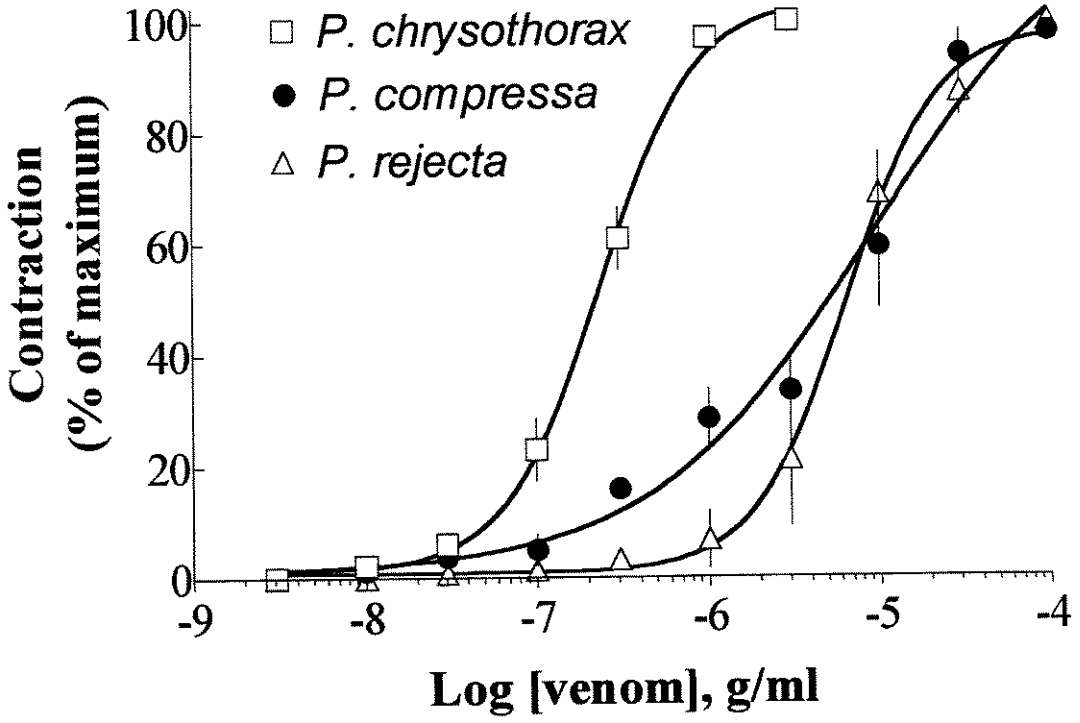

P. chrysothorax
P. compressa
P. rejecta
Contraction (% of maximum)
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log [venom], g/ml

Figure 1

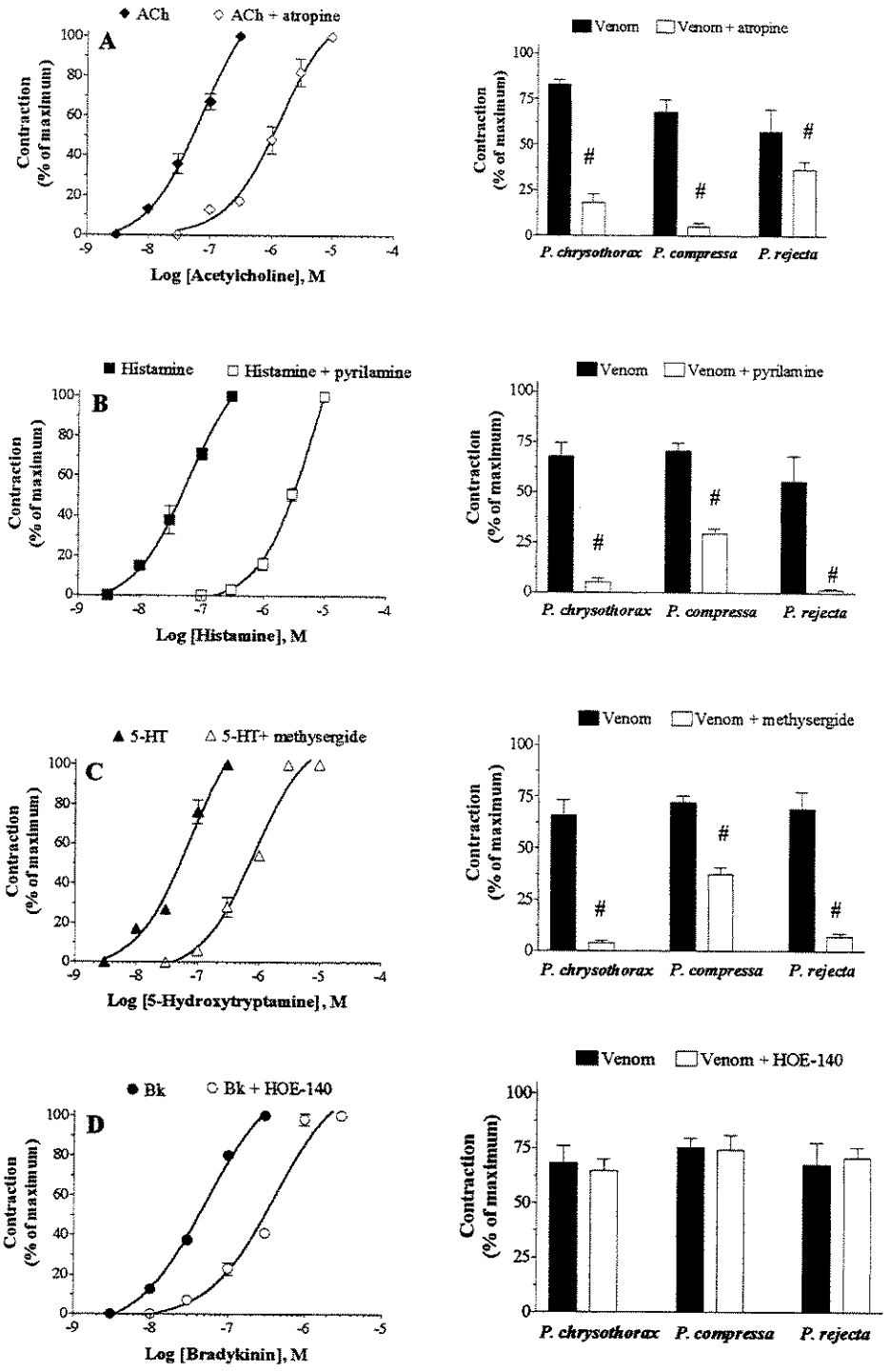

A
ACh
ACh + atropine
Contraction (% of maximum)
Log [Acetylcholine], M
Venom
Venom + atropine
P. chrysothorax
P. compressa
P. rejecta
B
Histamine
Histamine + pyrilamine
Log [Histamine], M
Venom + pyrilamine
C
5-HT
5-HT+ methysergide
Log [5-Hydroxytryptamine], M
Venom + methysergide
D
Bk
Bk + HOE-140
Log [Bradykinin], M
Venom + HOE-140

Figure 2

2

Cardiotoxicity of venoms from the Brazilian social wasps *Polybia chrysothorax*, *Polybia rejecta* and *Pseudopolybia compressa* in cockroach semi-isolated heart

CARDIOTOXICITY OF VENOMS FROM THE BRAZILIAN SOCIAL WASPS *Polybia chrysothorax, Polybia rejecta* AND *Pseudopolybia compressa* IN COCKROACH SEMI-ISOLATED HEART

Ericleison Cardoso-Silva[1], Sandro José Cordeiro[2] and Stephen Hyslop[1]

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

and

[2]Centro de Ciências Exatas e da Natureza,
Fundação de Ensino Superior de Olinda (FUNESO),
53060-770, Olinda, PE, Brasil

Running title: Cardiotoxicity of vespid venoms

Author for correspondence: Dr. Stephen Hyslop, Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), CP 6111, 13083-970, Campinas, SP, Brasil. Tel. 55-19-3788-9536, Fax: 55-19-3289-2968, E-mail: hyslop@fcm.unicamp.br

Abstract

The cardiotoxic action of venom from three species of social wasps (*Polybia chrysothorax, Polybia rejecta* and *Pseudopolybia compressa*) from northeastern Brazil was examined in the cockroach semi-isolated heart preparation. The venoms of *P. chrysothorax* and *P. compressa* produced a dose-dependent decrease in heart rate, with complete cardiac arrest within 15 min after the highest dose (100 μg); *P. rejecta* venom caused only a moderate reduction in heart rate at the highest dose. The cardiotoxicity of the two most active venoms was not attributable to the presence of acetylcholine, histamine, serotonin, bradykinin or phospholipase A_2 in these venoms. Venom PLA_2 may hastened the onset of cardiac arrest but was not directly responsible for it. Pretreating hearts with tetrodotoxin, a blocker of voltage-dependent sodium channels, delayed or prevented the venom-induced cardiac arrest. Glibenclamide, an antagonist of ATP-dependent potassium channels, enhanced the cardiotoxicity of *P. chrysothorax* venom but reduced that of *P. compressa*. 4-Aminopyridine, an antagonist of voltage-dependent potassium channels, delayed the onset of cardiac arrest, an effect which was greater for *P. compressa* venom. Nifedipine and verapamil, antagonists of voltage-dependent L-type calcium channels, had little or no effect on venom cardiotoxicity. These results indicate that *P. chrysothorax* and *P. compressa* venoms produce cardiac arrest by acting mainly on sodium channels. ATP- and voltage-dependent potassium channels have a variable role in this response whereas L-type voltage-dependent calcium channels are not involved. The dissimilar responses observed for *P. chrysothorax* and *P. compressa* with regard to sodium and potassium channel blockade suggest that the two venoms may differ in the pathways they activate. These findings also indicate that these venoms may contain novel toxins that target voltage-dependent sodium

channels and that the cockroach sem-isolated heart is useful for the rapid screening of cardiotoxicity in arthropod venoms.

Key words: cardiotoxic, cockroach, insecticidal, ion channels, phospholipase A_2, wasp venom

1. Introduction

Invertebrate bioassays, particularly from crustaceans and insects, have been widely used to screen and study the toxicity of arthropod venoms. Most studies have focused on the neurotoxicity of these venoms and their purified toxins, often with the view to their potential use as biopesticides (Piek, 1991; Eldefrawi *et al.*, 1993; Blagbrough *et al.*, 1994; Zlotkin, 1999). In contrast, relatively few studies have examined cardiotoxicity as a mechanism for the toxic activity of arthropod venoms and toxins. The cockroach (semi-) isolated heart is a simple, rapid, and reproducible bioassay that is useful for the rapid screening of biological activity. This preparation has been used extensively in studies of insect endocrinology and physiology (Miller and Metcalf, 1968; Miller *et al.*, 1979; Brian *et al.*, 1989; Predel *et al.*, 1999; Vilaplana *et al.*, 1999). Curiously, the usefulness of this preparation has generally been overlooked in the field of toxinology, despite the demonstration by Klowden *et al.* (1992) of its applicability in the rapid screening of cardiotoxicity in snake venom toxins. In this work, we have used the cockroach semi-isolated heart to examine the cardiotoxicity of venoms from three species of social wasps (*Polybia chrysothorax, Polybia rejecta* and *Pseudopolybia compressa*) from northeastern Brazil.

2. Materials and methods

2.1 Reagents

Acetylcholine, 4-aminopyridine, bradykinin, glibenclamide, histamine, indomethacin, nifedipine, pyrilamine, serotonin, tetrodotoxin, verapamil and phospholipase substrate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents of analytical grade were obtained from J.T. Baker (Xalostoc, Mexico), Mallinckrodt (Paris, KY, USA) or Merck (Rio de Janeiro, RJ, Brazil).

2.2 Venom collection

Colonies of the social wasps *Polybia chrysothorax*, *Polybia rejecta* and *Pseudopolybia compressa* were collected in the coastal forest zone of the state of Pernambuco in northeastern Brazil. The insects were killed with CO_2 and stored to -20°C until used. When required, the wasps were thawed on ice and the venom sacs removed along with the sting. Following removal of the sting, the venom was obtained by gently pressing (without squashing) the venom sacs in a beaker to expel their liquid content after which the sacs were stirred gently in distilled water with a magnetic stirrer at 4° C to ensure maximum recovery of venom. The venom sacs were subsequently removed by centrifugation (4200 rpm, 10 min) and the supernatant then recovered, lyophilized and stored to -20° C until required. The protein content of the material thus obtained was determined by the method of Lowry as modified by Peterson (1977), using bovine serum albumin as a standard. All amounts of venom mentioned below refer to this protein content.

2.3 Insects

Cockroaches (*Leucophea madere*) were housed at room temperature in a glass aquarium with free access to water and food (rodent Purina® chow).

2.4 Phospholipase activity

Phospholipase activity was measured using the assay described by Holzer and Mackessy (1996) modified for 96-well plates (Beghini *et al.*, 2000). The increase in absorbance at 425 nm after venom addition was monitored at 5 min intervals over a 30 min period using a SpectraMax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA, EUA).

2.5 Cockroach semi-isolated heart

The cockroach semi-isolated heart was used to examine the cardiotoxicity of the three venoms. The preparation was mounted essentially as described elsewhere (Bauman and Gersch, 1982; Klowden *et al.*, 1992; Predel *et al.*, 1999; Vilaplana *et al.*, 1999). Cockroaches were anesthetized with chloroform and immobilized with the ventral surface upwards. With the aid of a stereoscopic microscope, the abdominal cuticle was removed to reveal the internal organs. The digestive tract and other viscera were displaced to expose the heart which was then bathed in 0.15 M NaCl at room temperature. After allowing 5 min for the heart rate to stabilize, venom (1, 10 or 100 μg in 10 μl of saline, corresponding to concentrations of 0.1, 1 and 10 mg/ml) was applied to the heart and the changes in heart rate were then monitored over the following 30 min. Heart rate was recorded continuously

throughout the experiment using a video camera mounted on the eyepiece of the microscope.

When required, tetrodotoxin (TTX, 10^{-4} M; voltage-dependent sodium channel blocker), glibenclamide or 4-aminopyridine (10^{-4} M; potassium channel blockers), verapamil or nifedipine (10^{-4} M; calcium channel blockers) or indomethacin (10^{-3} M; cyclooxygenase inhibitor) was added to the preparations 15 min before the application of agonist or venom. Changes in heart rate were then recorded for a further 15 min. The agonist and antagonist concentrations used here were based on those reported in studies with other arthropod venoms.

2.6 Statistical analysis

The results were expressed as the mean ± S.E.M. Statistical comparisons were done using Student's unpaired t-test or ANOVA followed by the Tukey test. A value of $p<0.05$ indicated statistical significance.

3. Results

Figure 1 shows the effect of the three venoms on the beating rate of cockroach semi-isolated hearts. *P. chrysothorax* and *P. compressa* venoms showed marked cardiotoxicity which varied with the amount of venom applied. Rapid (within 4-12 min) and irreversible (by washing with 0.15 M saline) cessation of heart beat occurred with 100 µg of venom. In contrast, the venom of *P. rejecta* produced only a moderate reduction in heart rate, even with the highest amount of venom tested, and showed no dose-dependence.

Prior to investigating the mechanisms responsible for this cardiotoxicity, we examined the stability of the preparation in different solutions: 0.15 M NaCl, Tyrode solution and a cockroach or insect Ringer (Baumann and Gersch, 1982; Predel *et al.*, 1999; Vilaplana *et al.*, 1999). As shown in Fig. 2A, the preparation was stable for at least 30 min in all three solutions and the cardiotoxicity of *P. chrysothorax* venom was unaffected by the composition of the bathing solution (Fig. 2B). These findings agree with experiments in which the effect of varying concentrations of ions (up to 100 mM for K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}) on heart rate was examined in 0.15 NaCl; no changes in heart rate were seen with high or low concentrations of any of these ions (results not shown). These data indicate that the cardiotoxic action of the venoms was unlikely to be influenced the ionic composition of the "physiological" solution. In all experiments described below, 0.15 M NaCl was used to bathe the heart.

Wasp venoms contain acetylcholine, histamine, serotonin, and peptides, particularly kinins. Figure 3 shows the effect of several of these mediators on heart rate. Acetylcholine produced a slight increase in heart rate which rapidly returned to basal levels, whereas histamine had no effect. Serotonin produced a progressive decrease in heart rate without recovery in the 30 min period of observation and bradykinin produced a rapid decrease in heart rate that persisted for a few minutes before returning to basal levels. The amount of each agonist tested was 100 μg to correspond to the 100 μg of venom used, assuming that all of the venom consisted only of each particular agonist.

Social wasp venoms frequently contain high levels of phospholipase (PLA) which may be PLA_1, PLA_2, or PLB. As shown in Fig. 4, the venoms of *P. chrysothorax* and *P. compressa* had PLA levels comparable to those of rattlesnake (*Crotalus durissus terrificus*)

venom (positive control); the activity of *P. rejecta* venom was considerably lower. Figure 5 shows that bee (*Apis mellifera*) venom PLA_2 produced cardiac arrest which was abolished by pretreating the heart with indomethacin. However, indomethacin only delayed the onset but did not prevent the cardiotoxicity of the two most active venoms. This delay was particularly markd for *P. compressa* venom.

The involvement of ion channels in the cardiotoxicity of wasp venoms was examined by pretreating the hearts with various calcium, potassium and sodium channel antagonists. Figure 6 shows that tetrodotoxin (TTX, 10^{-4} M in 10 µl of saline), an antagonist of voltage-dependent sodium channels (Fozard e Hanck, 1996; Amat *et al.*, 1997; Ben Kalifa *et al.*, 1997) produced a decrease in heart rate which then stabilized at a level slightly below the basal rate. TTX completely prevented the decrease in heart rate caused by *P. chrysothorax* venom, in a manner similar to that seen with the marine toxin brevetoxin (PbTx-3), a sodium channel modulator (data not shown). In contrast, with *P. compressa* venom, TTX delayed the onset and rate of the decrease in heart rate but did not fully block the response.

The involvement of potassium channels was investigated by pretreating the hearts with glibenclemide, an antagonist of ATP-dependent potassium channels which prevents channels opening and leads to membrane depolarization, and 4-aminopyridine (4-AP), which blocks voltage dependent potassium channels and produces an effect similar to that seen with compounds which delay or prevent sodium channel inactivation.

Figure 7 shows that glibenclamide alone (10^{-4} M, dissolved in 2% DMSO; this concentration of DMSO had no effect on the heart responses) had no effect on heart rate. Glibenclamide slightly potentiated the response to *P. chrysothorax* venom and enhanced the cardiotoxic effect. For *P. compressa* venom, glibenclamide delayed the onset and severity of

the decrease in heart rate and offered about 50% protection. To examine whether the concomitant blockade of potassium and sodium channels would provide greater protection against the action of *P. compressa* venom than blockade of either alone, hearts were pretreated with glibenclamide and TTX (10^{-4} M each) prior to testing the venom. The response observed was the same as that seen with TTX alone, i.e. potassium channel inhibition did not summate with the effects of TTX (results not shown). In contrast to the effects of glibenclamide, 4-AP (10^{-3} M) initially delayed the onset of cardiotoxicity of both venoms, with a more pronounced action for *P. compressa* venom (Fig. 7).

The role of voltage-dependent L-type calcium channels in the venom-induced effects was investigated by pretreating the preparations with verapamil or nifedipine (10^{-4} M each) (Pelzer *et al.*, 1990; Wegener and Nässel, 2000; MacPherson *et al.*, 2001). As shown in Fig. 8, voltage-dependent L-type calcium channels are apparently not involved in the cardiac response to the venoms since the antagonists used had no effect on the decrease in heart rate, except for a slight delay in the full onset of cardiac arrest with nifedipine. Subsequent experiments using Bay K-8644 (10^{-4} M), an agonist selective L-type calcium channels (Tsien *et al.*, 1986; Pelzer *et al.*, 1990), suggested that cockroach may not contain L-type channels since this agonist had no effect on heart rate. Alternatively, these channels may be present but have different selectivities from their mammalian counterparts.

None of the pharmacological interventions described above had any effect on the slight decrease in frequency produced by *P. rejecta* venom (results not shown).

4. Discussion

The principal finding of this study was that the venoms of *P. chrysothorax* and *P. compressa* are cardiotoxic to the cockroach heart. This effect was dose-dependent, with cardiac arrest occurring rapidly at the highest amount of venom tested. This response is unlikely to be mediated by amines or kinins in the venoms. Two potential mediators (acetylcholine and histamine) had little effect on the heart rate. The progressive decrease in heart rate seen with the high amount (100 μg) of serotonin tested did not occur with lower quantities (≤50 μg). In other species of cockroach (*Periplaneta americana*), acetylcholine and serotonin cause excitation (an increase in heart rate) at micromolar concentrations or less (compared to the mM range used here) (Traina *et al.*, 1976; Miller, 1979; Baumann and Gersch, 1982; Predel *et al.*,1999; Vilaplana *et al.*, 1999). This variation problably reflects species and slight methodological differences in the type of preparation use. Although these venoms contain acetylcholine, histamine and serotonin (Cardoso-Silva *et al.*, submitted), their concentration is considerably below that tested here and hence have no significant action on the heart. Similarly, although bradykinin had a marked transitory effect on heart rate, this peptide is not responsible for the cardiotoxicity since these venoms do not contain kinins (Cardoso-Silva *et al.*, submitted).

Wasp venoms contain PLA_1 (King *et al.*, 1984; Schmidt *et al.*, 1986; Ho and Ko, 1988; Vargas-Villarreal *et al.*, 1991), PLA_2 (Oliveira and Palma, 1998; Costa and Palma, 2000) and PLB (King *et al.*, 1984; Takasaki and Fukumoto, 1989). Phospholipase A_2 produces smooth muscle contraction, hemolysis (Oliveira and Palma, 1998), mast cell degranulation (Habermann, 1972) and neurotoxicity (Edery *et al.*, 1972). However, as shown here, PLA contributed little to the cardiotoxicity of the venoms as seen in the

experiments with indomethacin, although the inhibition of PLA did delay somewhat the onset of cardiac arrest. In agreement with this, we have observed that the PLA-containing fraction of these venoms obtained by reverse phase HPLC had no effect on heart rate (results not shown), in contrast to the response observed with bee venom PLA_2.

The principal mechanism of cardiotoxicity appears to involve an action on voltage-dependent sodium channels since TTX, a specific blocker of these channels (Fozard and Hanck, 1996; Amat *et al.*, 1997; Ben Kalifa *et al.*, 1997), prevented the cardiac arrest by *P. chrysothorax* venom and markedly delayed that of *P. compressa*. Brevetoxin, produced by the marine dinoflagellate *Ptychodiscus brevis*, acts on sodium channels by delaying channel inactivation, thereby prolonging the channel open time (Richards *et al.*, 1990; Jeglitsch *et al.*, 1998). In cockroach heart, PbTx-3 (10^{-4} M) produced a decrease in heart rate, but not as marked as that seen with the venoms. This decrease was inhibited by TTX. These findings suggest that the venoms may act by influencing sodium channel gating and that this mechanism is inhibited by TTX.

The involvement of other ion channels in the venom-induced effects was less clear. Glibenclamide blocks ATP-dependent potassium channels and causes membrane depolarization. Treatment with glibenclamide markedly attenuated the cardiotoxicity of *P. compressa* venom, but potentiated that of *P. chrysothorax* venom, with a shift to the left in the time-response curve. This phenomenon may reflect the combined effects of increased excitability of the muscle fiber membrane following potassium channel blockade (with consequent action potential prolongation) and the inhibition of sodium channel inactivation by the venom. In the case of *P. compressa* venom, the protection offered by glibenclamide bore some semblance to that seen with TTX. However, when hearts were pretreated with

TTX and glibenclamide, the effect observed was the same as that of TTX alone rather than a summation of the two interventions. This finding suggests that the same physiological phenomenon is involved although different channels are being affected.

4-Aminopyridine blocks voltage-dependent potassium channels and prolongs the action potential of potassium during depolarization (Armstrong, 1981, 1992; Dreyer, 1990; Catterall, 1992; Pongs, 1992; Deal *et al.*, 1996). The effect of such blockade is similar to that seen with compounds that delay or inhibit sodium channel inactivation. Although 4-AP delayed the decrease in heart rate in the initial stages with both venoms (the protection was more marked with *P. compressa* venom), it did not prevent the final, complete cardiac arrest.

Voltage-dependent calcium channels do not appear to be involved since neither nifedipine nor verapamil had any marked effect on the cardiac arrest. Although slight shifts to the right were seen in the presence of these antagonists (especially nifedipine), they were not particularly marked and did not prevent cardiac arrest occurring within the same as the control hearts. It is possible that cockroach heart may not contain L-type voltage-dependent channels (or may have channels with different selectivities) since the selective agonist for these channels, Bay K 8644, had no effect on heart rate.

In conclusion, the venoms of *P. chrysothorax* and *P. compressa* contain toxins that are active on sodium channels. However, the discrepancies between the responses of these two venoms to sodium and potassium channel antagonists suggest that the mechanisms whereby they produce cardiac arrest are unlikely to be identical. The identification of the venom components responsible for this cardiotoxicity could provide novel compounds for studying sodium channels. Other ion channels and venom PLA_2 apparently contribute little

to this cardiotoxicity. These results also demonstrate the usefulness of the cockroach semi-isolated heart for screening arthropod venoms for cardiotoxins and provides a assay for detecting fractions with insecticidal activity during venom fractionation protocols.

Acknowledgments

The authors thank José Ilton dos Santos and Emanuela Barreto de Sousa for technical help, Dr. Edson Antunes (Departamento de Farmacologia, UNICAMP, Campinas, Brazil) for advice and for providing some of the drugs used in this study and Dr. Oton Meira Marques (Escola Politécnica de Agronomia, UFBA, Salvador, Bahia, Brazil) for identifying the wasps. E.C.S. was supported by a studentship from FAPESP (grant no. 00/05976-6). S.H. is supported by a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Amat C, Sinakevitch-Pean I, Hue B (1997) Proctolin-like immunoreactivty in dorsal paired media neurons generting plateau potecials in cockroach *Periplaneta americana*. *Neurosc. Res.* **29**, 263-268.

Armstrong CM (1992) Voltage-dependent ion channels and their gating. *Physiol. Rev.* **72**, S-5-13.

Armstrong CM (1981) Sodium channel and gating currents. *Physiol. Rev.* **61**, 644-683.

Baumann E, Gersch M (1982) Purification and identification of neurohormone D, a heart acelerating peptide from the corpora cardiaca of teh cockroach *Periplaneta americana*. *Insect Biochem.* **12 (1)**, 7-14.

Beghini DG, Tyama MH, Hyslop S, Sodek L, Novello JC, Maragoni S (2000) Enzymatic characterzation of a novel phospholipase A_2 from *Crotalus durissus cascavella* rattlesnake (Maracambóia) venom. *J. Protein. Chem.* **19 (7)**, 603-607.

Ben Kalifa R, Stankeiwicz M, Pelhates M, Serrano-Hernandez SE, Possani LD, Hinkel H, Mebs D (1997) Action of Babycurus-toxin 1 from the esat african scorpion *Babycurus centrurimorphus* on the isolated cockroach giant axon.Toxicon. **35 (7)**, 341-361

Blagbrough IS, Moya E, Taylor S (1994) Polyamines and polyamine amides from wasps and spiders. *Biochem. Soc. Trans.* **22,** 888-893.

Catterall WA (1992) Cellular and molecular biology of voltage-gated sodium channels. *Physiol. Rev.* **72**, S-15-48.

Costa H, Palma MS (2000) Agelotoxin: A phospholipase A_2 from the venom of the neotropical social wasp cassununga (*Agelaia pallipes pallipes*) (Hymenoptera – Vespidae). *Toxicon.* **38**, 1367-1379.

Deal KK, England SK, Tamkun MM (1996) Molecular physiology of cardiac potassium channels. *Physiol. Rev.* **76**, 49-67.

Dreyer F (1990) Peptide toxins and potassium channels. *Res.Physiol. Biochem. Pharmacol.* **115**, 94-140.

Edery H, Ishay J, Gitter S (1972) Pharmacologycal activity of oriental hornet (*Vespa orientalis*) venom. *Toxicon* **10,** 13-23.

Eldefrawi ME, Anis NA, Eldefrawi AT (1993) Glutamate receptor inhibitors as potential insecticides. *Arch. Insect Biochem. Physiol.* **22,** 25-39.

Fozzard HA, Hanck DA (1996) Structure and function of voltage-dependent sodium channel: comparison of brain II and cardiac isoforms. *Physiol. Rev.* **76**, 887-925.

Habermann E (1972) Bee and wasp venoms. *Science* **177**, 314-322.

Ho CL, Ko JL (1988) Purification and characterization of a letal protein with phospholipase A_1 activity from the hornet (*Vespa basalis)* venom. *Biochim. Biophys. Acta* **963**, 414-422.

Holzer M, Mackessy SP (1996) An aqueous endpoint assay of snake venom phospholipase A_2. *Toxicon*. **34**, 1149-1155.

Jeglistsch G, Rein K,Bades DG, Adams Dj (1990) Brevetoxin-3 (PbTx-3-3) and its derivates modulate single tetrodotoxin-sensitive sodium channel in rat sensory neurons. *Pharmacol.* **284**, 516-525.

King TP, Kochoumian L, Jolyn A (1984) Wasp venom proteins: Phospholipase A_1 and B. *Arch. Biochem. Bioph.* **230 (1)**, 1-12.

Klowden MJ. Vitale AJ, Trumble MJ., Wessan CR, Trumble WR. (1992) A bioassay for cobra cardiotoxin activity using semi-isolated cockroach heart. *Toxicon.* **30**, 295-301.

MacPherson MR, Pollock VP, Broderick KE, Kean L, O'Connell FC, Dow JAT, Cavies SA (2001) Model organisms: New insights into ion channel and transporter function. L-type calcium channels regulate epithelial fluid transport in *Drosophila malenogaster. Am. J. Physiol. Cell. Physiol.* **280**, 394-415.

Miller T, Metcalf rl (1968) Site of action of pharmacologically actve compounds on the heart of *Periplaneta americana* L. *J Insect Physiol.* **14**,:383-394.

Miller T (1979) Nervous versus neurohormonal control of insect heartbeat. *Am. Zool.* **19**, 77-86.

Oliveira MR, Palma MS (1998) Plytoxins: a group of phospholipases A_2 from the venom of the neotropical social wasp paulistinha (*Polybia paulista*). *Toxicon.* **36**, 189-199.

Pelzer D, Pelzer S, MacDonald TF (1990) Properties and regulation of calcium channel in muscle cells. *Rev. Physiol. Biochem.* **114**, 108-207.

Peterson GL (1977) A simplification of the protein assay methodof Lowriy *et al.* Which is more generally applicable. Annal Biochem. 83, 357-363.

Piek T (1991) Neurotoxic kinins from wasp and ant venoms. *Toxicon* **29,** 139-149.

Predel R, Kellner R, Rapus J, Nachman R, Gäde G (1999) Post-translational modifications of the insect sulfakinins. *Eur. J. Biochem.* **263**, 552-560.

Richards IS, Kulkarni AP, Brooks SM, Pierce R (1990) Florida red-toxins (brevetoxins) produce depolarization of airway smooth muscle. *Toxicon.* **28**, 1105-1111.

Schmidt JO, Blum MS, Overal WL (1986) Comparativeenzymology of venoms from stinging hymenoptera. *Toxicon.* **24 (9)**, 907-921.

Takasaki C, Fukumoto M (1989) Phospholipase B from venom japae yellow hornet (*Vespa xanthotera*) venom. *Toxicon.* **24 (4)**, 449-485.

Traina ME, Bellino M, Serpietri L, Massa A, Frontali N (1976) Heart-accelerating peptides from cockroach corpor cardiaca. *J. Insect Physiol.* **22**, 323-329.

Tsien RW, Bean BP, Hess P, Lansman JB, Nilius B, Nowycky MC (1986) Mechanisms ofcalcium channel modulation by b-adrenergic agents and dihydropyridine calcium agonists. *J. Mol. Cell Cardiol.* **18**, 691-710.

Vargas-Villarreal J, Martin-Polo J, Reynaud E, Alagon AC (1991) A new affinity adsorbent for the purification of phospholipases A_1 and A_2 from animal venoms. *Toxicon.* **29 (1)**, 119-124.

Vilaplana L, Maestro JL, Piulachs M-D, Bellés X (1999) Modulation of cardiac rhythm by allostatins in the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *J. Insect Physiol.* **45**, 1057-1064.

Wegener C, Nässel DR (2000) Peptide-induced Ca^{2+} moviments in a tonic insect muscle: effects of proctolin and periviscerokinin-2. *J. Neurophysiol.* **84**, 3056-3075.

Zlotkin E (1999) The insect voltage-gated sodium channel as target of insecticides. *Annu. Rev. Entomol.* **44,** 429-455.

FIGURE LEGENDS

Figure 1. Cardiotoxic activity of (A) *P. chrysothorax*, (B) *P. compressa* and (C) *P. rejecta* venoms in cockroach semi-isolated heart. The preparations were mounted as described in Methods. The points are the mean±S.E.M. of four hearts for each amount of venom indicated.

Figure 2. Stability of the cockroach semi-isolated heart preparation in different physiological solutions (A), and lack of influence of the ionic composition of these solutions on the cardiotoxicity of *P. chrysothorax* venom (100 μg). The preparations were mounted as described in Methods. The composition of the Ringer solution was (in g/l): NaCl 9, KCl 0.8, $NaHCO_3$ 0.8, $CaCl_2$ 0.8, and that of the Tyrode solution: NaCl 8.19, KCl 0.37, $MgCl_26H_2O$ 0.2, $CaCl_2$ 0.56, glucose 2.4, HEPES 2.4. The points are the mean±S.E.M. of three hearts.

Figure 3. Effect of (A) acetylcholine and bradykinin and (B) histamine and serotonin on heart rate in cockroach semi-isolated hearts. A fixed quantity of each agonist (100 μg) was tested in each case. The points are the mean±S.E.M. of 3-4 hearts.

Figure 4. Phospholipase activity of the wasp venoms studied. Activity was determined at described in Methods. Rattlesnake (*Crotalus durissus terrificus*) snake venom was used as a positive control.

Figure 5. Effect of indomethacin (10^{-3} M) on the decrease in cockroach heart rate caused by the venoms of *P. chrysothorax* and *P. compressa*. (A) Bee venom PLA_2 (5 μg), (B) *P. chrysothorax* venom (100 μg), and (C) *P. compressa* venom (100 μg). The points are the mean±S.E.M. of 3-4 hearts

Figure 6. Influence of tetrodotoxin (TTX, 10^{-4} M) on the cardiotoxicity of (A) *P. chrysothorax* and (B) *P. compressa* venoms (100 μg each). The points are the mean±S.E.M. of 3-4 hearts.

Figure 7. Influence of glibenclamide (10^{-4} M) and 4-aminopyridine (10^{-4} M) on the cardiotoxicity of (A) *P. chrysothorax* and (B) *P. compressa* venoms (100 μg each). The points are the mean±S.E.M. of 3-4 hearts.

Figure 8. Influence of verapamil (10^{-4} M) and nifedipine (10^{-4} M) on the cardiotoxicity of (A) *P. chrysothorax* and (B) *P. compressa* venoms (100 μg each). The points are the mean±S.E.M. of 3-4 hearts.

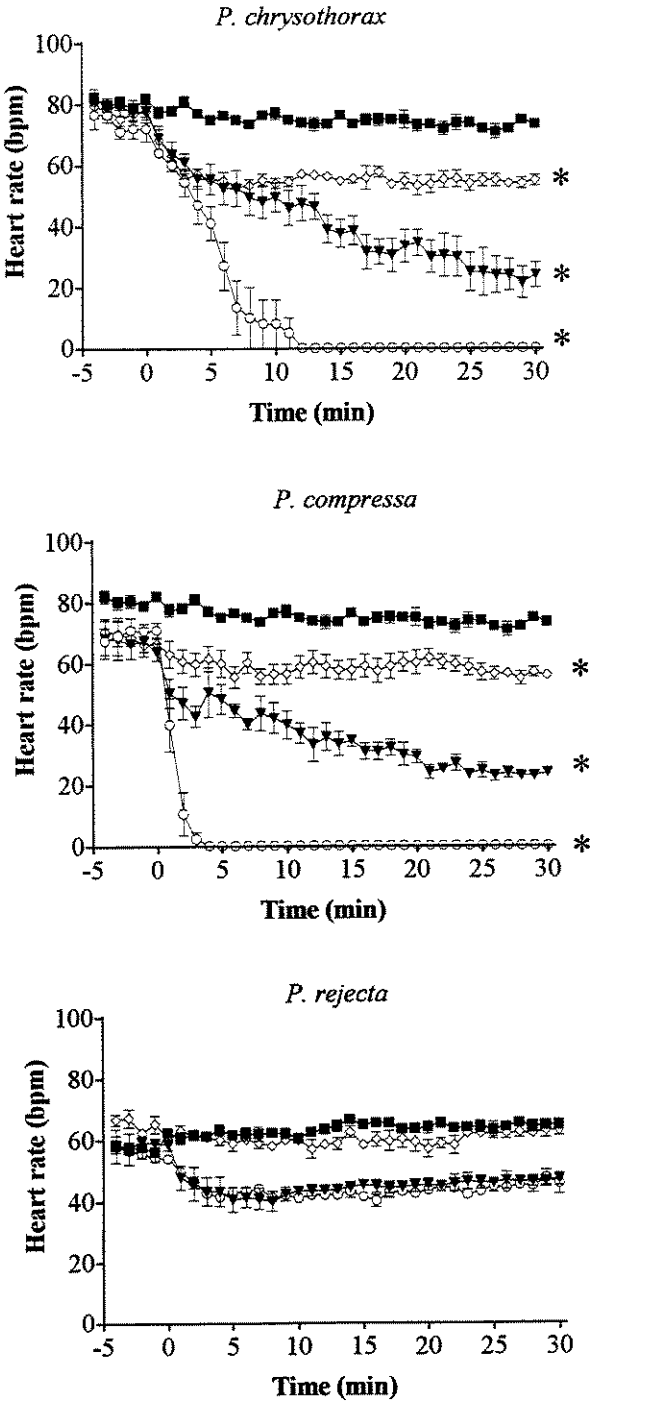
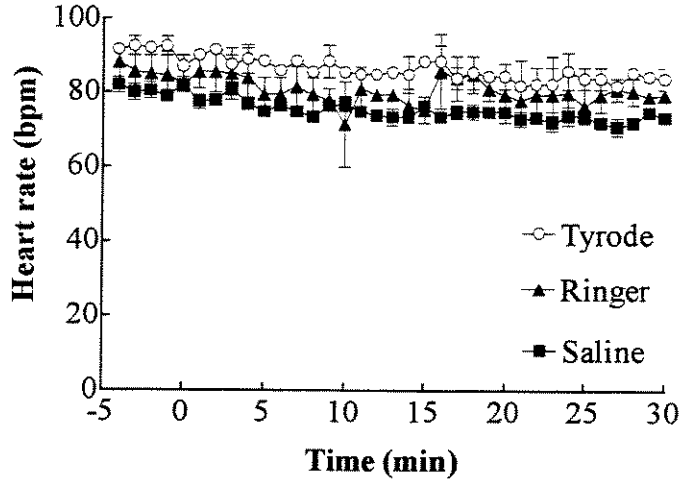

P. chrysothorax
Heart rate (bpm)
Time (min)
P. compressa
Heart rate (bpm)
Time (min)
P. rejecta
Heart rate (bpm)
Time (min)
Control
1 µg
10 µg
100 µg

Figure 1


Heart rate (bpm)
100
80
60
40
20
0
-5
0
5
10
15
20
25
30
Time (min)
Tyrode
Ringer
Saline

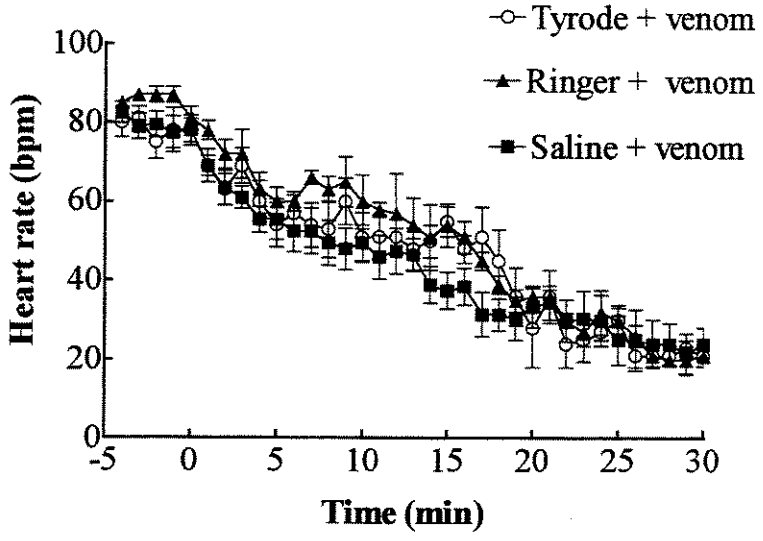

Heart rate (bpm)
100
80
60
40
20
0
-5
0
5
10
15
20
25
30
Time (min)
Tyrode + venom
Ringer + venom
Saline + venom

Figure 2

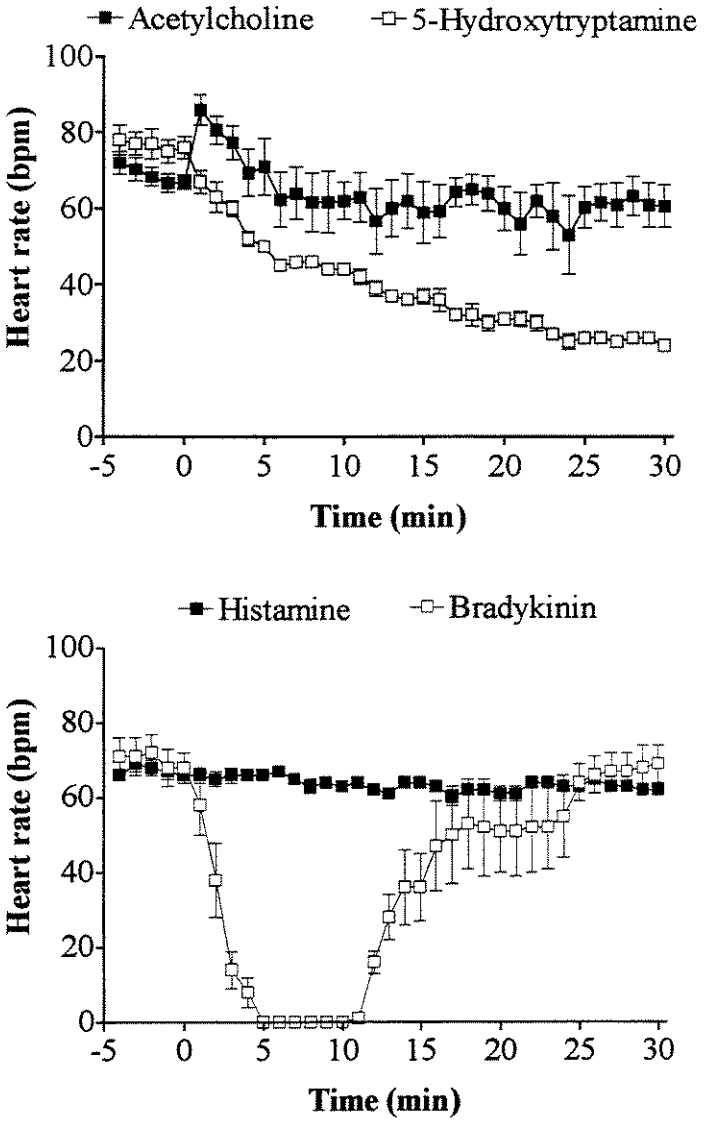

Acetylcholine
5-Hydroxytryptamine
Heart rate (bpm)
100
80
60
40
20
0
-5
0
5
10
15
20
25
30
Time (min)
Histamine
Bradykinin
Heart rate (bpm)
100
80
60
40
20
0
-5
0
5
10
15
20
25
30
Time (min)

Figure 3

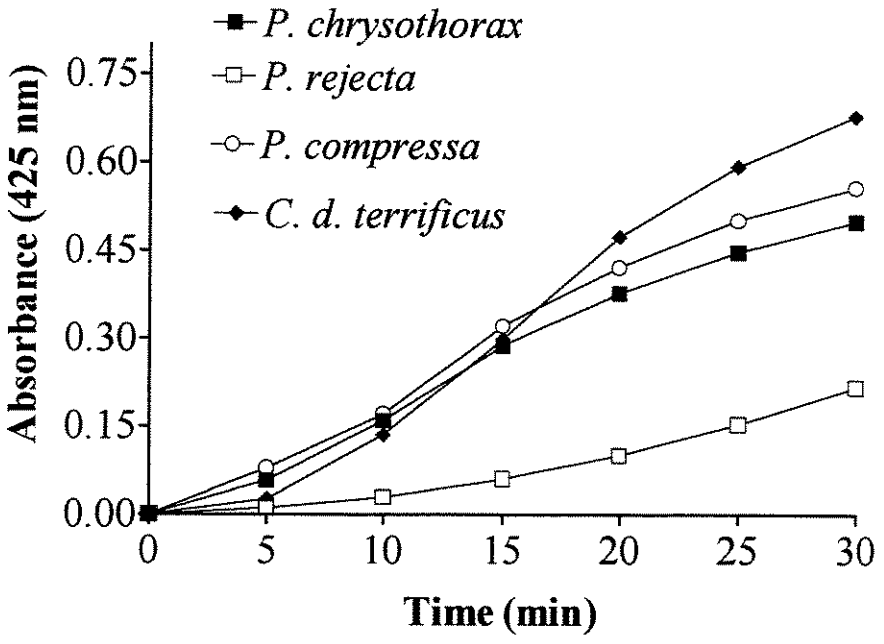
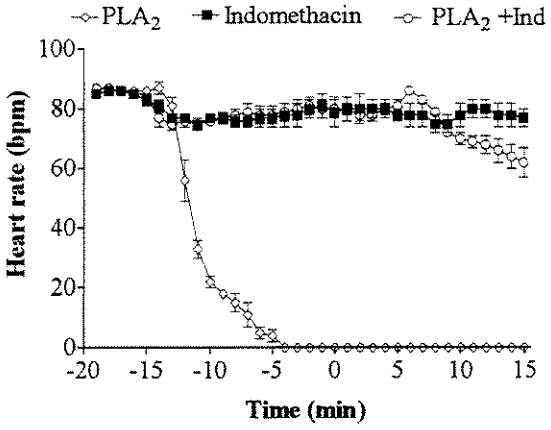
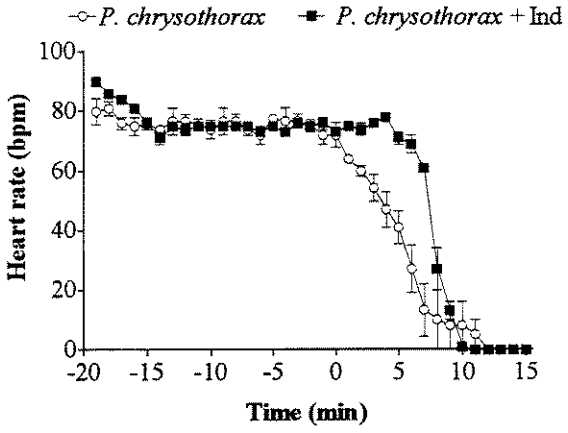

P. chrysothorax
P. rejecta
P. compressa
C. d. terrificus
Absorbance (425 nm)
0.75
0.60
0.45
0.30
0.15
0.00
0
5
10
15
20
25
30
Time (min)

Figure 4


PLA2
Indomethacin
PLA2 +Ind
Heart rate (bpm)
100
80
60
40
20
0
-20
-15
-10
-5
0
5
10
15
Time (min)


P. chrysothorax
P. chrysothorax + Ind
Heart rate (bpm)
100
80
60
40
20
0
-20
-15
-10
-5
0
5
10
15
Time (min)

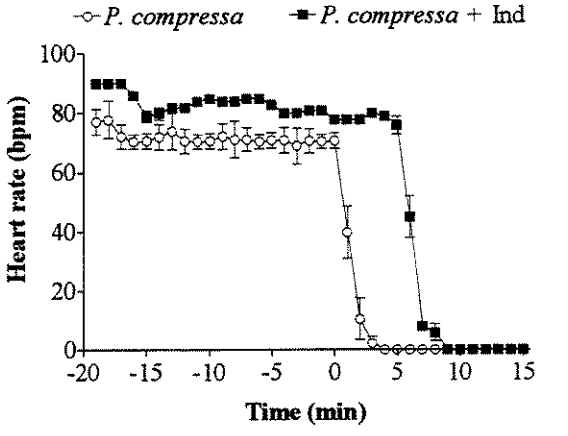

P. compressa
P. compressa + Ind
Heart rate (bpm)
100
80
60
40
20
0
-20
-15
-10
-5
0
5
10
15
Time (min)

Figure 5

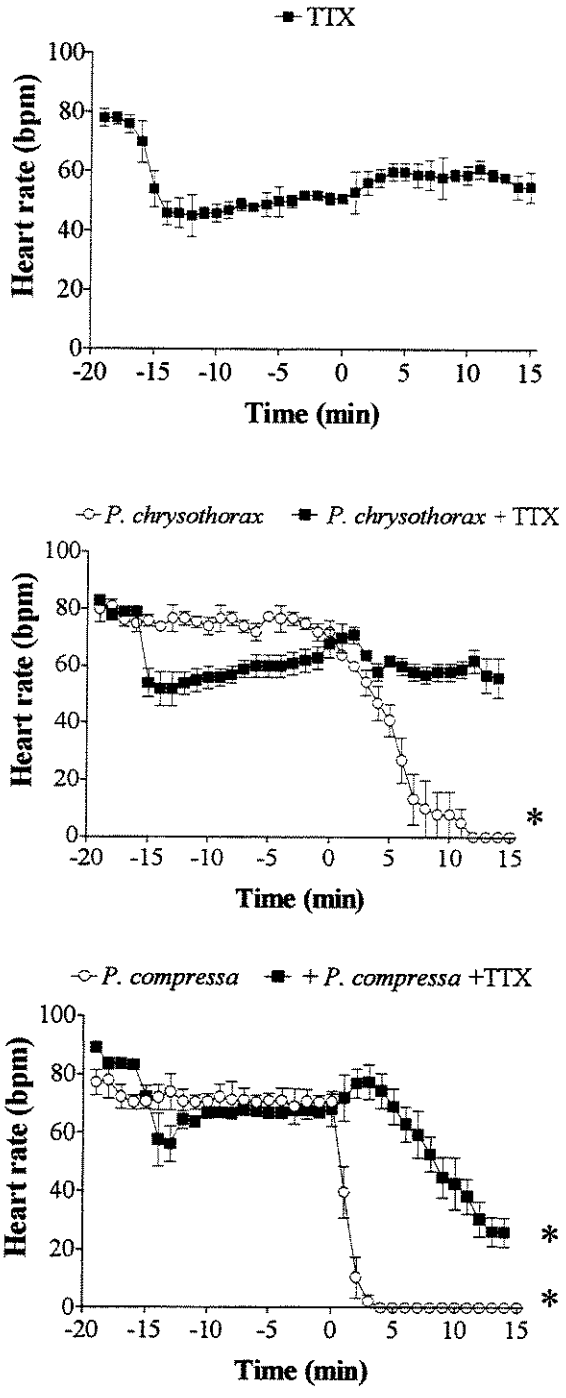

TTX
Heart rate (bpm)
Time (min)
P. chrysothorax
P. chrysothorax + TTX
Heart rate (bpm)
Time (min)
*
P. compressa
+P. compressa +TTX
Heart rate (bpm)
Time (min)
*
*

Figure 6

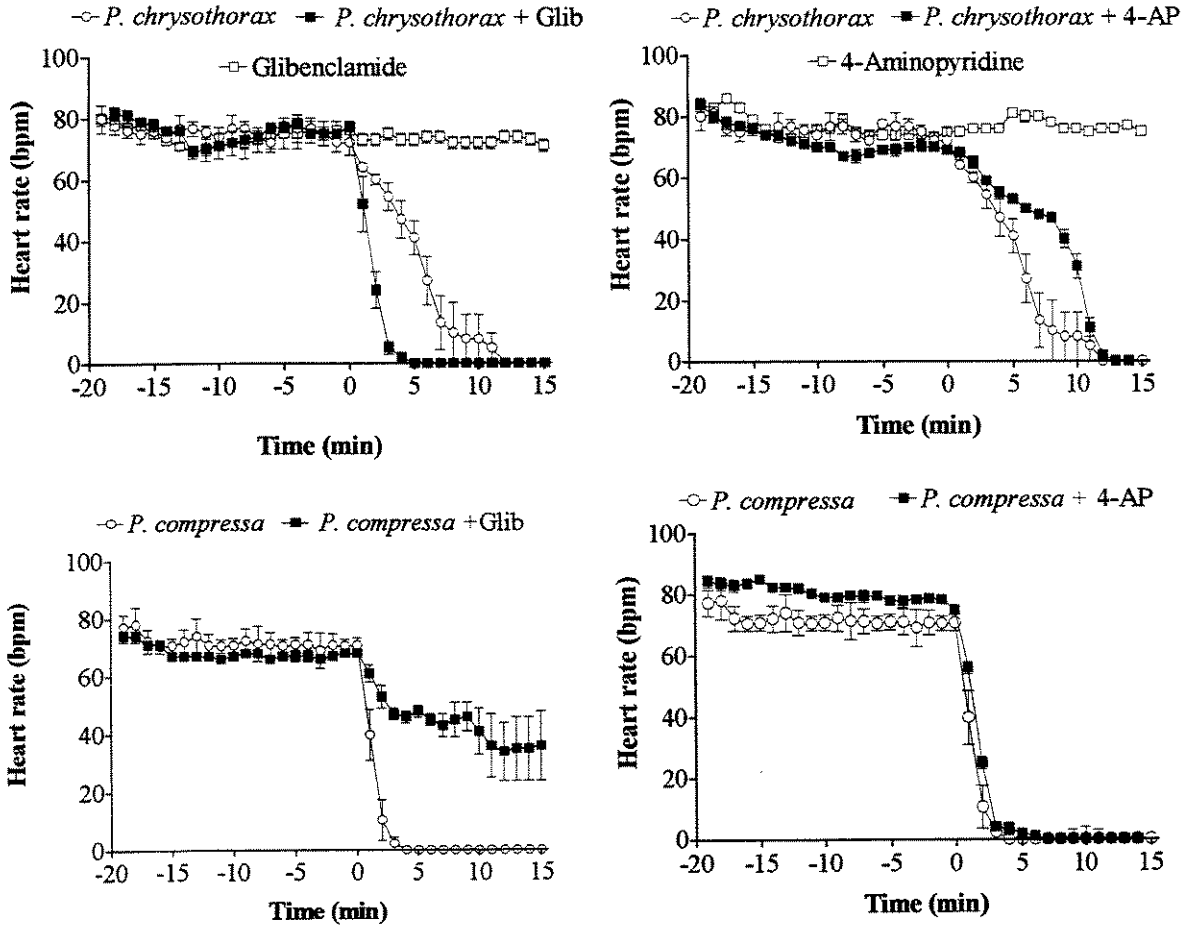

P. chrysothorax
P. chrysothorax + Glib
Glibenclamide
Heart rate (bpm)
Time (min)
P. chrysothorax
P. chrysothorax + 4-AP
4-Aminopyridine
Heart rate (bpm)
Time (min)
P. compressa
P. compressa +Glib
Heart rate (bpm)
Time (min)
P. compressa
P. compressa + 4-AP
Heart rate (bpm)
Time (min)

Figure 7

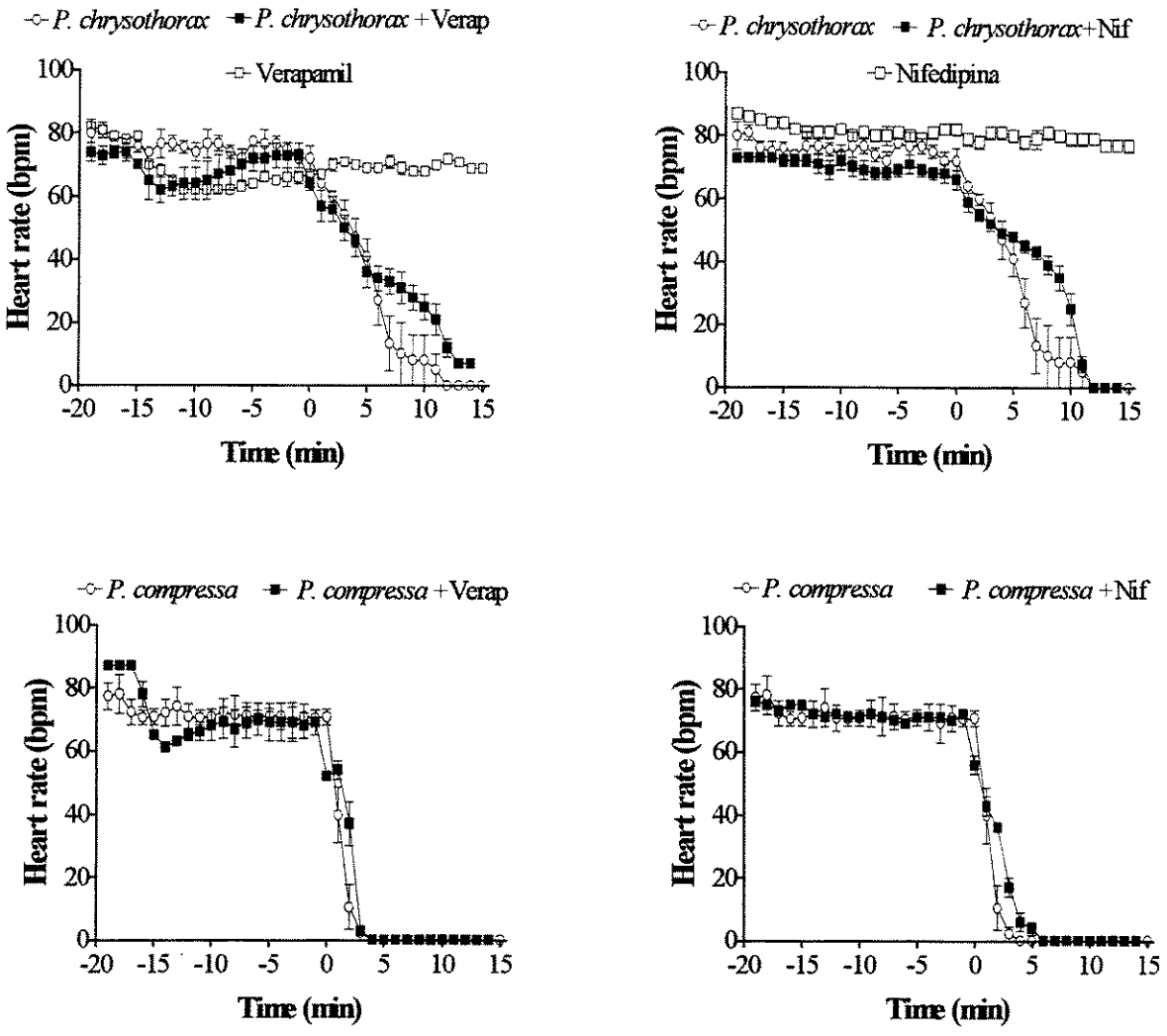

P. chrysothorax
P. chrysothorax +Verap
Verapamil
Heart rate (bpm)
Time (min)
P. chrysothorax
P. chrysothorax+Nif
Nifedipina
Heart rate (bpm)
Time (min)
P. compressa
P. compressa +Verap
Heart rate (bpm)
Time (min)
P. compressa
P. compressa +Nif
Heart rate (bpm)
Time (min)

Figure 8

3

Sodium channel involvement in the neurotoxicity and cardiotoxicity of venom from the social wasp *Polybia chrysothorax* in arthropod preparations: identification of a pompilidotoxin-like peptide

SODIUM CHANNEL INVOLVEMENT IN THE NEUROTOXICITY AND CARDIOTOXICITY OF VENOM FROM THE SOCIAL WASP *Polybia chrysothorax* IN ARTHROPOD PREPARATIONS: IDENTIFICATION OF A POMPILIDOTOXIN-LIKE PEPTIDE

Ericleison Cardoso Silva[1], Marcos Hikari Toyama[2], Sergio Marangoni[2], José Carlos Freitas[3] and Stephen Hyslop[1,*]

[1]Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), CP 6111, 13083-970, Campinas, SP, Brasil.

[2]Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, 13083-970, Campinas, SP, Brasil.

[3]Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo (USP), São Paulo, SP, Brasil

Running title: Wasp venom and sodium channels

***Author for correspondence**: Dr. Stephen Hyslop, Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), CP 6111, 1308-970, Campinas, SP, Brasil. Tel. (55)-(19)-3788-9536. Fax: (55)-(19)-3289-2968. E-mail: hyslop@fcm.unicamp.br

Abstract

The effect of venom from the social wasp *Polybia chrysothorax* from northeastern Brazil on nerve action potentials of the blue crab *Callinectes danae* and on heart rate in cockroach semi-isolated heart preparations was examined. Mechanical stimulation of the terminal leg appendage (dactylus) of *C. danae* was used to produce action potentials in the corresponding sensory nerve. In this preparation, *P. chrysothorax* venom (100 μg) caused a discharge of action potentials independent of stimulation. This effect was similar to that seen with brevetoxin-3 (PbTx-3, $5x10^{-7}$ M), a marine toxin which activates sodium channels acting on site 5. The effect of the venom was prevented by tetrodotoxin (TTX, 10^{-6} M), which blocks sodium channels. In sucrose-gap experiments using electrically stimulated sensory nerves, *P. chrysothorax* venom (10, 30 and 100 μg) markedly reduced the action potential amplitude without affecting the duration. The ED_{50} for the venom-induced blockade of action potential transmission in this preparation was 25.1 ± 0.3 μg (mean ± SEM, n=3, $p<0.05$). PbTx-3 ($5x10^{-7}$ M) and TTX (10^{-6} M) also blocked action potential generation in this preparation. In cockroach heart, the venom (100 μg) caused irreversible cardiac arrest which was prevented by TTX (10^{-4} M). Fractionation of the venom by reverse phase HPLC yielded a principal peak containing a peptide with the sequence ARSLLEGLGIRRGSA, which showed strong homology with α- and β-pompilidotoxins. This toxin reproduced the neurotoxic and cardiotoxic actions of the venom. These results indicate that the venom of *P. chrysothorax* contains a toxin that acts on voltage-dependent sodium channels, probably by activating or delaying their inactivation.

Key words: nerve, action potential, sodium channel, wasp venom.

1. Introduction

Wasp venoms contain a variety of substances, including low molecular weight compounds such as amino acids (Abe *et al.*, 1989) and amines (catecholamines, dopamine, histamine, and 5-hydroxytryptamine) (Edery *et al.*, 1978), as well as peptides, particularly kinins (Edery *et al.*, 1978; Piek, 1984; 1991; Konno *et al.*, 2002) and mastoparans (Ho *et al.*, 1994; Chuang *et al.*, 1996; Nakajima *et al.*, 1998). In addition, enzymes such as hyaluronidase (Allalouf *et al.*, 1972; Edery *et al.*, 1978) and phospholipase A (Vargas-Villarreal *et al.*, 1991; Ho and Ko, 1988; Oliveira and Palma, 1998; Costa and Palma, 2000) occur in these venoms.

In recent years, various studies have shown that wasp venoms contain a range of neurotoxic compounds, some of which are insect-selective. The best characterized of these are kinins and their derivatives which, in addition to their smooth muscle contracting activity, also block cholinergic neurotransmission in insect preparations (reviewed in Piek, 1991). Other compounds identified include polyamines such as philanthotoxin (Piek, 1982, 1984; Kits and Piek, 1986; Bruce *et al.*, 1990; Blagbrough *et al.*, 1994) and peptides known as α– and β–pompilidotoxins (α– and β–PMTXs) from the venoms of the solitary wasps *Anoplius samariensis* and *Batozonellus maculifrons*, respectively (Konno *et al.*, 1998), which delay the inactivation of sodium channels (Konno *et al.*, 2002; Sahara *et al.*, 2000; Kinoshita *et al.*, 2001; Miyawaki *et al.*, 2002). Other toxins, such as mandaratoxin (MDTX) from *Vespa mandarinia* (Abe *et al.*, 1982), also act by selectively blocking sodium currents. The venom of the social wasp *Vespa insularis* contains a toxin that opens chloride channels (Hori *et al.*, 1977; Kawai *et al.*, 1979).

Neurotoxins active on ion channels, such as polyamines and PMTXs, have been isolated primarily from solitary wasp species, with comparatively little being known about their occurrence

in social wasps. In this report, we describe the neurotoxic and cardiotoxic actions of venom from the social wasp *Polybia chrysothorax* from northeastern Brazil using crustacean and insect preparations, and show that the effects of the venom are mediated by a toxin active on voltage-dependent sodium channels.

2. Material and methods

2.1. Reagents

Brevetoxin-3 and tetrodotoxin were from Sigma Chemical Co. (St. Louis, MO, USA) and the salts for physiological solutions were from J.T. Baker (Xalostoc, Mexico), Mallinckrodt (Paris, KY, USA) or Merck (Rio de Janeiro, RJ, Brazil).

2.2. Venoms

Colonies of the social wasp *Polybia chrysothorax* were collected in the coastal forest zone of the state of Pernambuco in northeastern Brazil. The insects were killed with CO_2 and stored to -20°C until used. When required, the wasps were thawed on ice and the venom sacs removed along with the sting. Following removal of the sting, the venom was obtained by gently pressing (without squashing) the venom sacs in a beaker to expel their liquid content after which the sacs were stirred gently in distilled water with a magnetic stirrer at 4° C to ensure maximum recovery of venom. The venom sacs were subsequently removed by centrifugation (4500 rpm, 10 min) and the supernatant then recovered, lyophilizad and stored to -20° C until required. The protein content of the material thus obtained was determined by the method of Lowry modified by Peterson (1977) using bovine serum albumin as standard. All amounts of venom mentioned below refer to this protein content.

2.3. Reverse phase high performance liquid chromatography (RP-HPLC)

Venom was dissolved in 0.5% (v/v) trifluoroacetic acid and applied to a C_{18} reverse phase column (4 x 250 mm, 5 μm, AmershamBiosciences) preequilibrated with 0.5% TFA. After washing to remove unbound material, proteins were eluted with a linear gradient (0-65%) of 60% acetonitrile in 0.5% TFA at a flow rate of 1 ml/min using an ÄKTApurifier 10 chromatography system. The elution profile was monitored at 280 nm and fractions were collected manually.

2.4. Amino acid sequence determination

The amino acid sequence of the active peak obtained by HPLC was determined by direct Edman degradation using a Procise f automated sequencer.

2.5. Sensory nerve action potentials following mechanoreceptor stimulation

This preparation was mounted essentially as described by Freitas *et al.* (1992) using adult specimens of the blue crab *Callinectes danae* collected in the São Sebastião Channel on the northern coast of the state of São Paulo. The distal portion of the second or third pair of legs was removed together with its corresponding nerve and transferred to a recording chamber mounted in a Faraday cage to eliminate electrical interference. The extremity of the leg was exposed through an orifice in the chamber and positioned to allow stimulation by filtered sea water dripped from a height of 15 cm. The nerve bundle passed through a small chamber (volume, 100 μl) to which the venom or drug was added and was then connected to a suction electrode. The nerve was bathed in physiological solution (composition, in mM: NaCl 470.4, KCl 8.0, $CaCl_2.2H_2O$ 18.0, $MgCl_2.6H_2O$ 1.5, $NaHCO_3$ 6.0, glucose 5.6) at room temperature. Action potentials in response to mechanoreceptor stimulation with dripping sea water were recorded with a Grass model P55 AC

pre-amplifier via an analogical-digital interface coupled to a computer loaded with the software Whole Cell Electrophysiology Program (WCP) version 1.2 (Digidata 1200, Axon Instruments, Union City, CA, USA). The recordings were also displayed on a Tektronix 5103 oscilloscope. After mounting, the preparations were allowed to stabilize for 15 min before the application of venom or toxin in 10 µl of physiological solution. Control recordings were obtained before the application of test substance and for up to 15 min thereafter. The concentrations of venom and toxins used are indicated in the Results and figure legends.

2.6. Sucrose gap recordings

The preparation was obtained essentially as described above except that before mounting the nerve was separated from the leg and then mounted in an 8-compartment acrylic chamber according to Malpezzi *et al.* (1993). The acrylic chamber consisted of platinum-iridium stimulation electrodes connected to compartments 1 (positive) and 2 (negative), respectively, while the silver chloride recording electrodes were located in compartments 5 and 8. Compartments 1 and 5 contained physiological solution (composition as above), compartments 6 and 7 contained 1 M sucrose and compartment 8 contained 0.5 M KCl. The venom and toxins were diluted in physiological solution and applied to the fifth compartment.

Electrodes placed before and after the sucrose gap recorded the ionic current passing through the nerve fiber during evoked action potentials. Supramaximal stimuli (0.2 mV) were delivered from a Grass SD-9 stimulator. The potentials, which were amplified via a high impedance DC preamplifier (model NF-1, Bioelectric Instruments) and displayed on a Tektronix 5103 oscilloscope, were recorded on computer disk via an analogical-digital interface using the software Whole Cell Electrophysiology Program (WCP) version 1.2.

2.7. Cockroach semi-isolated heart

The cockroach semi-isolated heart was used to examine the cardiotoxicity of *P. chrysothorax* venom and its purified toxin. The preparation was mounted essentially as described elsewhere (Bauman and Gersch, 1982; Klowden *et al.*, 1992; Predel *et al.*, 1999; Vilaplana *et al.*, 1999). Cockroaches (*Leucophea madere*) were anesthetized with chloroform and immobilized with the ventral surface upwards. With the aid of a stereoscopic microscope, the abdominal cuticle was removed to reveal the internal organs. The digestive tract and other viscera were displaced to expose the heart which was then bathed in 0.15 M NaCl at room temperature. After allowing 5 min for the heart rate to stabilize, *P. chrysothorax* venom (100 μg) or toxin (purified toxin – 6.4 x 10^{-3} M, PbTx-3 - 10^{-4} M or TTX - 10^{-4} M) was applied to the heart in 10 μl of saline and the changes in heart rate were then monitored over the following 30 min. Heart rate was recorded continuously throughout the experiment using a video camera mounted on the eyepiece of the microscope. When required, TTX was applied to the heart 15 min before testing the venom, purified toxin or PbTx-3 (quantities and concentrations given above). Thereafter, heart rate was measured for a further 15 min.

2.8. Statistical analysis

The results were expressed as the mean ± S.E.M., as appropriate. Statistical comparisons were done using Student's unpaired t-test or ANOVA followed by the Tukey test. A value of $p<0.05$ indicated statistical significance.

3. Results

3.1. Evoked action potentials following mechanoreceptor stimulation

Figure 1A shows the control response of *C. danae* sensory nerve to mechanical stimulation, in which the induced action potential discharge rapidly disappeared after stimulation. Figure 1B (left panel) shows that PbTx ($5x10^{-7}$ M) increased the frequency of action potentials shortly after application, independent of mechanoreceptor stimulation. When the full effects of PbTx-3 had occurred, the potentials normally seen in response to mechanical stimulation were no longer detectable (Fig. 1B, right panel). *P. chrysothorax* venom (100 μg) also increased the frequency of sensory nerve potentials shortly after application and independent of mechanical stimulation (Fig. 1C). However, the speed of onset and intensity of the effect were slightly less than observed with PbTx-3 (Fig. 1B, both panels). Washing the preparations did not reverse the increased neural excitability seen with the venom (results not shown).

Figure 2 shows that the sodium channel blocker TTX abolished the action potentials in response to mechanoreceptor stimulation. TTX also reversed the effects of PbTx-3 and the toxin purified from *P. chrysothorax*, although this reversal required some time (~1 min) to occur.

3.2. Sucrose gap recordings

In sucrose gap recordings, *P. chrysothorax* venom (100 μg) blocked sensory nerve action potentials evoked by electrical stimulation (Fig. 3A). This blockade occurred 2-5 min after the application of venom and was dose-dependent, with an ED_{50} of 25.1 ± 0.3 μg (n=3) (Fig. 3B). Brevetoxin-3 (PbTx-3, $5x10^{-7}$ M) and tetrodotoxin (TTX, 10^{-6} M) also blocked the action potentials (Fig. 3A).

3.3. Cockroach semi-isolated heart

The application of PbTx-3 (10^{-4} M) reduced the heart rate by more than 50% (Fig. 4A). TTX (10^{-4} M) alone also reduced the heart rate. However, the decrease in heart rate with TTX was not as marked as seen with PbTx-3 and gradually returned to normal or stabilized at a frequency below the pre-toxin rate. Neither of these toxins produced complete cardiac arrest at the concentrations tested. Pre-treating hearts with TTX abolished the decrease in heart rate normally seen with PbTx-3. As shown in Fig. 4B, *P. chrysothorax* venom (100 μg) produced rapid (within 15 min) cardiac arrest in the cockroach semi-isolated heart; this reponse was dose-dependent and irreversible by washing (data not shown). Pre-treating the hearts with TTX virtually abolished the cardiotoxic action of *P. chrysothorax* venom.

3.4. Identification of a pompilidotoxin-like peptide in P. chrysothorax venom

To identify the venom component responsible the neurotoxic and cardiotoxic effects described above, *P. chrysothorax* venom was fractionated by RP-HPLC on a C_{18} column. Figure 5A shows the elution profile obtained for the venom. Although several peaks were observed, only the principal one (indicated by the arrow) showed biological activity. This peak contained a single peptide, the sequence of which as determined by direct automated sequencing is shown in Fig. 5B. A search of protein sequence databases provided matches only with α- and β-pompilidotoxins isolated from the venoms of the solitary wasps *Anoplius samariensis* and *Batozonellus maculifrons*, respectively (Konno *et al.*, 1998). This peptide, which we have tentatively named γ-pompilidotoxin (γ-PMTX), MW= 1555.8, reproduced the neurotoxic (Figs. 1D, 2D and 3B) and cardiotoxic (Fig. 4C) activities of the venom described above. As with the venom, the effects of the toxin were abolished by pretreating the preparations with TTX.

4. Discussion

Voltage-dependent sodium channels are involved in the generation of action potentials in excitable tissues (Armstrong, 1992; Catterall, 1992, 2000; Fozzard and Hanck, 1996), and this central role in electrophysiology makes them an important target for a variety of toxins from animal venoms, particularly in insects (Zlotkin, 1999). Toxins which act by preventing or retarding the inactivation of sodium channels have been identified in scorpion (Pelhate e Zlotkin, 1982; Rogers *et al.*, 1996; Ben Kalifa *et al.*, 1997; Sautière *et al.*, 1998; Li *et al.*, 2000) and spider (Rash and Hodgson, 2002) venoms, but also include marine toxins such as PbTx (Richards *et al.*, 1990; Schreibmayer and Jeglitsch, 1992; Jeglitsch *et al.*, 1998) and solitary wasp venom toxins such as α– and β–pompilidotoxins (α-PMTX and β-PMTX) (Konno *et al.*, 1997, 1998; Sahara *et al.*, 2000). Other toxins, such as TTX and saxitoxin (STX), act as true channel blockers (Catterall, 1993; Fozzard and Hanck, 1996).

The results described here indicate that the venom of the social wasp *P. chrysothorax* acts on voltage-dependent sodium channels in crustacean sensory nerve fibers and in cockroach heart. The neural effects of *P. chrysothorax* venom were similar to those seen with PbTx-3, a lipophilic molecule produced by the marine dinoflagellate *Ptychodiscus brevis* (=*Gymnodinium breve*). PbTx has a high affinity for site 5 of voltage-dependent sodium channels. This toxin depolarizes excitable membranes and prolongs the opening of sodium channels, thereby increasing the influx of sodium ions and causing "bursts" of activity (Richards *et al.*, 1990; Schreibmayer and Jeglitsch, 1992; Jeglitsch *et al.*, 1998). Blockade of neurotransmission is eventually produced by overexcitation of the tissue. In cardiac and smooth muscle, this depolarization can result in muscle contraction (Richards *et al.*, 1990). These responses are abolished by the sodium channel blocker TTX.

The similarity between the effects of the venom and PbTx-3 suggested that the former might contain a toxin active on voltage-dependent sodium channels and that its mechanism of action might involve the prolongation of sodium channel inactivation. The presence of such a toxin was confirmed by the finding that only one fraction obtained following RP-HPLC reproduced the cardiotoxic and neurotoxic activities of the venom. Sequencing of the material in this peak yielded γ-PMTX, a peptide with strong homology to α– and β–pompilidotoxins (α– and β–PMTXs), 13-amino acid peptides isolated from the venoms of the solitary wasps *Anoplius samariensis* and *Batozonellus maculifrons*, respectively (Konno *et al.*, 1998).

As shown in Fig. 5, the principal difference in the sequence of γ-PMTX compared to the other two toxins is the presence of three additional amino acids at the C-terminal. Six amino acid residues (Ser_3, Leu_4, Leu_8, Gly_9, Ile_{10} and Arg_{13}) are conserved in the three peptides. In addition, there are conservative substitutions, i.e., no change in amino acid side chain polarity or charge, at positions 1, 2, 6, 7 and 8. These conservative changes have retained the positive, negative and positive residues at positions 2, 6 and 11, respectively. This finding agrees with the structure-function study of Konno *et al.* (2000) who reported that conservation of the charged residues at positions 1, 11 and 13 (positions 12, 3 and 1, respectively, in their nomenclature) was fundamental for biological activity (repetitive action potentials in lobster leg preparations). The only difference between the sequences of α-PMTX and β-PMTX is the presence of arginine instead of lysine at position 2 in β-PMTX. This single change makes β-PMTX 5-7 times more potent in the lobster leg preparation (Konno *et al.*, 1998, 2000). Interestingly, γ-PMTX also contains arginine at this same position.

In their studies, Konno *et al.* (2000) and Sahara *et al.* (2000) also showed that substitutions at various other positions in the peptides, including aspartic acid at position 6,

resulted in partial or total loss of activity. These findings indicate that while some residues may be important for binding to specific sites in sodium channels, all are required to provide the correct three-dimensional conformation of the peptide. This conclusion is supported by the observation that fragments of α-PMTX containing 4-9 amino acids were devoid of activity. The effect of additional amino acid residues beyond position 13 (present in γ-PMTX) was not investigated.

Pompilidotoxins affect neurotransmission by acting on neurons to delay sodium channel inactivation, generally without affecting the rate or peak of channel activation. This action involves slowing or blocking the conformational changes required for inactivation and leads to hyperexcitability of the tissue and consequent blockade of neurotransmission (Konno *et al.*, 1997; Sahara *et al.*, 2000). The ability of *P. chrysothorax* venom and γ-PMTX to cause hyperexcitability was seen in the crab sensory nerve preparation. Our observations that the actions of γ-PMTX were prevented by TTX agree with those of Sahara *et al.* (2000) who reported that α-PMTX acted preferentially on TTX-sensitive sodium channels and had no effect on TTX-resistant sodium channels of rat trigeminal ganglion neurons. In addition to their action in invertebrate preparations, α-PMTX and β-PMTX also affect sodium channel inactivation and neurotransmission in the mammalian central nervous system (Harsch *et al.*, 1998; Sahara *et al.*, 2000; Yokota *et al.*, 2001; Miyawaki *et al.*, 2002).

As shown here, γ-PMTX interacted with neural and cardiac sodium channels of invertebrates. Because of the limited amount of purified material available, it was not possible to compare the potencies of this toxin in the two preparations. Nevertheless, the fact that γ-PMTX affected sodium channels in both tissues differs from the findings of Kinoshita *et al.* (2001) who reported that β-PMTX slowed the inactivation of rat brain sodium channels but not those of rat heart. This difference was attributed to the presence of an acidic residue (Glu1616) in the

extracellular loop of D4S3-S4 in brain sodium channels compared to the homologous residue (Gln1615) in heart sodium channels since producing a Glu1616Gln mutant in brain channels abolished sensitivity to β-PMTX whereas doing the reverse in heart channels (Gln1615Glu) yielded channels sensitive to the toxin. These findings, together with the small size of pompilidotoxins (13-16 amino acids) compared to scorpion α-toxins (60-65 amino acids) and sea anemone toxins (46-49 amino acids) which also slow sodium channel inactivation (Catterall, 1995), suggest that perhaps only a small region of the latter two groups of toxins interacts directly with the receptor site (Kinoshita *et al.*, 2001). Whether similar selectivities and sensitivities to PMTXs exist amongst invertebrate voltage-dependent sodium channels remains to be determined.

In conclusion, *P. chrysothorax* venom blocks neurotransmission and produces cardiac arrest in arthropods by acting on cardiac and neuronal sodium channels perhaps slowing channel inactivation and produce a state of membrane hyperexcitability. This effect is also mediated principally by a novel member of the pompilidotoxin peptide family. This is the first demonstration of such a toxin in social wasp venoms and indicates that pompilidotoxins may have a widespread distribution in the venoms of social and solitary wasps. Detailed electrophysiological studies are now required to compare γ-PMTX with the other PMTXs and to assess the extent to which its structural peculiarities influence its biological activities.

Acknowledgments

The authors thank José Ilton dos Santos for technical assistance, Dr. Edson Antunes (Departamento de Farmacologia, UNICAMP) for providing tetrodotoxin and brevetoxin, Dr. Oton Meira Marques (Escola Politécnica de Agronomia, UFBA, Salvador, Bahia, Brazil) for identifying the wasps, and the staff of the Centro de Biologia Marinha (CEBIMAR), São

Sebastião, SP, for providing access to laboratory facilities and accommodation. E.C.S. was supported by a studentship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant no. 00/05976-6). S.H. is supported by a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Abe T, Hariya Y, Kawai N, Niwa A (1989) Comparative study of amino acid composition in an extract from hornet venom sacs: high content of neuroactive amino acids in *Vespa*. *Toxicon* **27**, 683-688.

Abe T, Kawai A (1983) Cardioactive effects of hornet venom (*Vespa mandarinia*). *Comp. Biochem. Physiol.* **76**, 221-228.

Abe T, Kawai N, Niwa A (1982) Purification and properties of a presynaptically acting neurotoxin, mandaratoxin, from hornet (*Vespa mandarinia*). *Biochemistry* **21,** 1693-1697.

Allalouf D, Ber a, Ishay J (1972) Hyaloronidase activity of extracts of venom sacs of a number of vespinae (hymenoptera). *Comp. Biochem. Physiol.* **43 B**, 119-123.

Armstrong CM (1992) Voltage-dependent ion channels and their gating. *Physiol. Rev.* **72**, S5-S13.

Baumann E, Gersch M (1982) Purification and identification of neurohormone D, a heart acelerating peptide from the corpora cardiaca of teh cockroach *Periplaneta americana*. *Insect Biochem.* **12 (1)**, 7-14.

Ben Kalifa R, Stankiewicz M, Pelhate M, Serrano-Hernandez SE, Possani LD, Hinkel H, Mebs D (1997) Action of babycurus-toxin 1 from the East African scorpion *Babycurus centrurimorphus* on the isolated cockroach giant axon. *Toxicon* **35**, 1069-1080.

Blagbrough IS, Moya E, Taylor S (1994) Polyamines and polyamine amides from wasps and spiders. *Biochem. Soc. Trans.* **22,** 888-893.

Catterall WA (1992) Cellular and molecular biology of voltage-gated sodium channels. *Physiol. Rev.* **72**, S15-S48.

Catterall WA (1993) Structure and function of voltage-gated ions channels. *Trends Neurosci.* **16**, 500-506.

Catterall WA (1995) Structure and function of voltage-gated ion channels. *Annu. Rev. Biochem.* **64,** 493-531.

Catterall WA (2000) From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* **26,** 13-25.

Costa H, Palma MS (2000) Agelotoxin: a phospholipase A_2 from the venom of the neotropical social wasp cassununga (*Agelaia pallipes pallipes*) (Hymenoptera – Vespidae). *Toxicon* **38**, 1367-1379.

Chuang CC, Huang WC, Yu HM, Wang KT, Wu SH (1996) Conformation of *Vespa basalis* mastoporan-B in trifluoroethanol-containing aqueous solution. *Biochim. Biophys. Acta* **1292**, 1-8.

Edery H, Ishay J, Gitter S, Joshua H (1978) Venoms of Vespidae. In: *Handbook of Experimental Pharmacology. Vol. 48. Arthopod Venoms.* (Bettini S, ed.). pp. 691-771. Spinger Verlag: Berlin.

Fozzard HA, Hanck DA (1996) Structure and function of voltage-dependent sodium channels: comparison of brain II and cardiac isoforms. *Physiol. Rev.* **76**, 887-926.

Freitas JC, Ogata M, Kodama M, Martinez SCG, Lima MF, Monteiro CK (1992) Possible microbial source of guanidine neurotoxins found in the mussel *Perna perna* (Mollusca, Bivalvia, Mytilidae). In: *Recent Advances in Toxinological Research. Vol. 2.* (Gopalakrishnakone P, Tan CK, eds.). pp. 589-596, National University of Singapore Press: Singapore.

Harsch A, Konno K, Takayama H, Kawai N, Robinson H (1998) Effects of α-pompilidotoxin on synchronized firing in networks of rat cortical neurons. *Neurosci. Lett.* **252,** 49-52.

Ho CL, Hwang LL, Lin YL, Chen CT, Yu HM, Wang KT (1994) Cardiovascular effects of mastoparan B and its structural requirements. *Eur. J. Pharmacol.* **259**, 259-264.

Ho CL, Ko JL (1988) Purification and characterization of a lethal protein with phospholipase A_1 activity from the hornet (*Vespa basalis)* venom. *Biochim. Biophys. Acta* **963**, 414-422.

Hori S, Kawai N, Niwa A, Ohtani S (1977) Separation of neurotoxins from hornet *(Vespa insularis)* venom and their actions on crustacean neuromuscular transmission. *J. Neurochem.* **28**, 1183-1188.

Jeglitsch G, Rein K, Baden DG, Adams DJ (1998) Brevetoxin-3 (PbTx-3) and derivates modulate single tetrodotoxin-sensivtive sodium channels in rat sensory neurons. *Pharmacol.* **284**, 516-525.

Kawai N, Abe T, Hori S, Niwa A (1979) Effects of a neurotoxin (HTX-E) in hornet venom on synaptic transmission. *Neurosci. Lett.* **2 (suppl.),** 13.

Kawai N, Abe T, Hori S, Niwa A (1980) Effects of neurotoxin in hornet venom on neuromuscular transmission of lobster. *Comp. Biochem. Physiol.* **65C,** 87-92.

Kits KS, Piek T (1986) Action of the polyamine β-philanthotoxin on neuromuscular transmission in insects. *Neuropharmacology* **25,** 1089-1093.

Konno K, Hisada M, Itagaki Y, Naoki H, Nobufumi K, Miwa A, Yasuhara T, Takayama H (1998) Isolation and structure of pompilidotoxins, novel peptide neurotoxins in solitary wasp venoms. *Biochem. Biophys. Res. Commun.* **250,** 612-616.

Konno K, Hisada M, Naoki H, Itagaki Y, Yasuhara T, Nakata Y, Miwa A, Kawai N (2000) Molecular determinants of binding of a wasp toxin (PMTXs) and its analogs in the Na^+ channels proteins. *Neurosci. Lett.* **285,** 29-32.

Konno K, Miwa A, Takayama H, Hisada M, Itagaki Y, Naoki H, Yasuhara T, Kawai N (1997) α-Pompilidotoxin (α-PMTX), a novel neurotoxin from the venom of a solitary wasp, facilitates transmission in the crustacean neuromuscular synapse. *Neurosci. Lett.* **238**, 99-102.

Klowden MJ. Vitale AJ, Trumble MJ., Wessan CR, Trumble WR. (1992) A bioassay for cobra cardiotoxin activity using semi-isolated cockroach heart. *Toxicon.* **30**, 295-301.

Li YJ, Tan ZY, Ji YH (2000) The binding of BmK IT2, a depressant insect-selective scorpion toxin, on mammal and insect sodium channels. *Neurosci. Res.* **38**, 257-264.

Malpezzi ELA, Freitas JC, Muramoto K, Kamiya H (1993) Characterzation of peptides in sea anemone venom collected by a novel procedure. *Toxicon* **31**, 853-864.

Miyawaki T, Tsubokawa H, Yokota H, Oguro K, Konno K, Masuzawa T, Kawai N (2002) Differential effects of novel wasp toxin on rat hippocampal interneurons. *Neurosci. Lett.* **328,** 25-28.

Oliveira MR, Palma MS (1998) Polybiatoxins: a group of phospholipases A_2 from the venom of the neotropical social wasp paulistinha (*Polybia paulista*). *Toxicon* **36**, 189-199.

Pelhate M, Zlotkin E (1982) Actions of insect toxin and other toxins derived from the venom of the scorpion *Androctonus australis* on isolated giant axons of the cockroach (*Periplaneta americana*). *J. Exp. Biol.* **97**, 67-77.

Piek T (1982) δ-Philanthotoxin, a semi-irreversible blocker of ion channels. *Comp. Biochem. Physiol.* **72C,** 311-315.

Piek T (1984) Pharmacology of Hymenoptera venoms. In: *Handbook of Natural Toxins. Vol. 2. Insect Poisons, Allergens and other Invertebrate Venoms.* (Tu AT, ed.). pp. 135-185. Marcel Dekker: New York.

Piek T (1991) Neurotoxic kinins from wasp and ant venoms. *Toxicon* **29,** 139-149.

Predel R, Kellner R, Rapus J, Nachman R, Gäde G (1999) Post-translational modifications of the insect sulfakinins. *Eur. J. Biochem.* **263**, 552-560.

Rash LD, Hodgson WC (2002) Pharmacology and biochemistry of spider venoms. *Toxcon* **40,** 225-254.

Richards IS, Kulkarni AP, Brooks SM, Pierce R (1990) Florida red-tide toxins (brevetoxins) produce depolarization of airway smooth muscle. *Toxicon* **28**, 1105-1111.

Rogers JC, Qu Y, Tanada TN, Scheuer T, Catterall WA (1996) Molecular determinants of high affinity binding of α-scorpion toxin and sea anemone toxin in the S3-S4 extracellular loop in domain IV of the sodium channel α subunit. *J. Biol. Chem.* **271**, 15950-15962.

Sahara Y, Gtoh M, Konno K, Miwa A, Tsubokawa H, Robinson HPC, Kawai N (2000) A new class of neurotoxin from wasp venom slows inactivation of sodium current. *Eur. J. Neurosci.* **12**, 1961-1970.

Sautière P, Cestèle S, Kopeya C, Martinage A, Drobecq H, Doljansky Y, Gordon D (1998) New toxins acting on sodium channels from the scorpion *Leiurus quinquestriatus hebraeus* suggest a clue to mammalian vs insect seletivity. *Toxicon.* **36**, 1141-1154.

Schreibmayer W, Jeglitsch G (1992) The sodium channel activator brevetoxin-3 uncovers a multiplicity of different open states of cardiac sodium channel. *Biochim. Biophys. Acta* **1104**, 233-242.

Vargas-Villarreal J, Martin-Polo J, Reynaud E, Alagon AC (1991) A new affinity adsorbent for the purification of phospholipases A_1 and A_2 from animal venoms. *Toxicon* **29**, 119-124.

Vilaplana L, Maestro JL, Piulachs M-D, Bellés X (1999) Modulation of cardiac rhythm by allostatins in the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *J. Insect Physiol.* **45**, 1057-1064.

Yasuhara T, Mantel P, Nakajima T, Piek T (1987) Two kinins isolated from an extract of the venom reservoirs of the solitary wasp *Megascolia flavifrons*. *Toxicon* **25**, 527-535.

Yokota H, Tsubokawa H, Miyawaki T, Konno K, Nakayama H, Masuzawa T, Kawai N (2001) Modulation of synaptic transmission in hippocampal CA1 neurons by a novel neurotoxin (β-pompilidotoxin) derived from wasp venom. *Neurosci. Res.* **41,** 365-371.

Yoshida H, Geller RG, Pisano JJ (1976) Vespulakinin: new carbohydrate-containing bradykinin derivates. *Biochemistry* **15**, 61-64.

Zlotkin E (1999) The insect voltage-gated sodium channel as target of insecticides. *Annu. Rev. Entomol.* **44,** 429-455.

FIGURE LEGENDS

Figure 1. Sensory nerve action potentials recorded during the stimulation of crab (*C. danae*) leg mechanoreceptors. (A) Control response to stimulation, and responses in the presence of (B) brevetoxin (PbTx-3, $5x10^{-7}$ M), (C) *P. chrysothorax* venom (100 μg) and (D) purified *P. chrysothorax* toxin, γ-PMTX (6.4×10^{-4} M). See text for experimental details. Arrow – addition of venom or toxin. Asterisk – mechanical stimulation of nerve. The mean time lapse between the left and right panels of each pair was ~0.8 min. The time and voltage scales in (A) are the same for the other panels.

Figure 2. Effect of tetrodotoxin (TTX) on the sensory nerve action potentials recorded during stimulation of crab (*C. danae*) leg mechanoreceptors. (A) Control response to stimulation, and responses to TTX (10^{-6} M) alone (B). (C) and (D) show the effect of TTX on the responses to brevetoxin (PbTx-3, $5x10^{-7}$ M) and *P. chrysothorax* toxin, γ-PMTX (6.4×10^{-4} M), respectively. See text for experimental details. Arrow – addition of venom or toxin. Asterisk – mechanical stimulation of nerve. Note that TTX (10^{-6} M) was added in the interval between the left and right recordings in each pair (mean time lapse between the left and right panels was ~0.8 min). The time and voltage scales in (A) are the same for the other panels.

Figure 3. Sucrose gap recordings from electrically stimulated *C. danae* sensory nerve. Panel A shows the inhibition of potentials by (1) *P. chrysothorax* venom (100 μg), (2) *P. chrysothorax* toxin, γ-PMTX (6.4×10^{-4} M), (3) TTX (10^{-6} M) and (4) PbTx-3 (5×10^{-7} M). Panel B shows the

dose-dependence of the inhibition by *P. chrysothorax* venom. The points are the mean±S.E.M. of three experiments. See text for experimental details.

Figure 4. Cardiotoxic action of *P. chrysothorax* venom in cockroach semi-isolated heart and its inhibition by TTX (10^{-4} M). (A) Brevetoxin (PbTx-3, 10^{-4} M), (B) *P. chrysothorax* venom (100 μg), (C) *P. chrysothorax* purified toxin, γ-PMTX (6.4×10^{-4} M). Note that pretreating the hearts with TTX abolished the cardiotoxic action of all the venom and two toxins. The points are the mean±S.E.M. of 4 experiments.

Figure 5. Elution profile of *P. chrysothorax* venom following RP-HPLC and amino acid sequence of the toxin (γ-PMTX) responsible for the cardiotoxic and neurotoxic activities of the venom. The arrow in the elution profile indicates the peak corresponding to the sequenced toxin. In the table, the sequences of α– and β–pompilidotoxins are shown for comparison. Note that a space has been inserted at position 12 in the sequence for γ-PMTX to obtain greater homology. Bold letters – conserved residues. Underlined letters – conservative substitutions in the γ-PMTX sequence (see text for details).

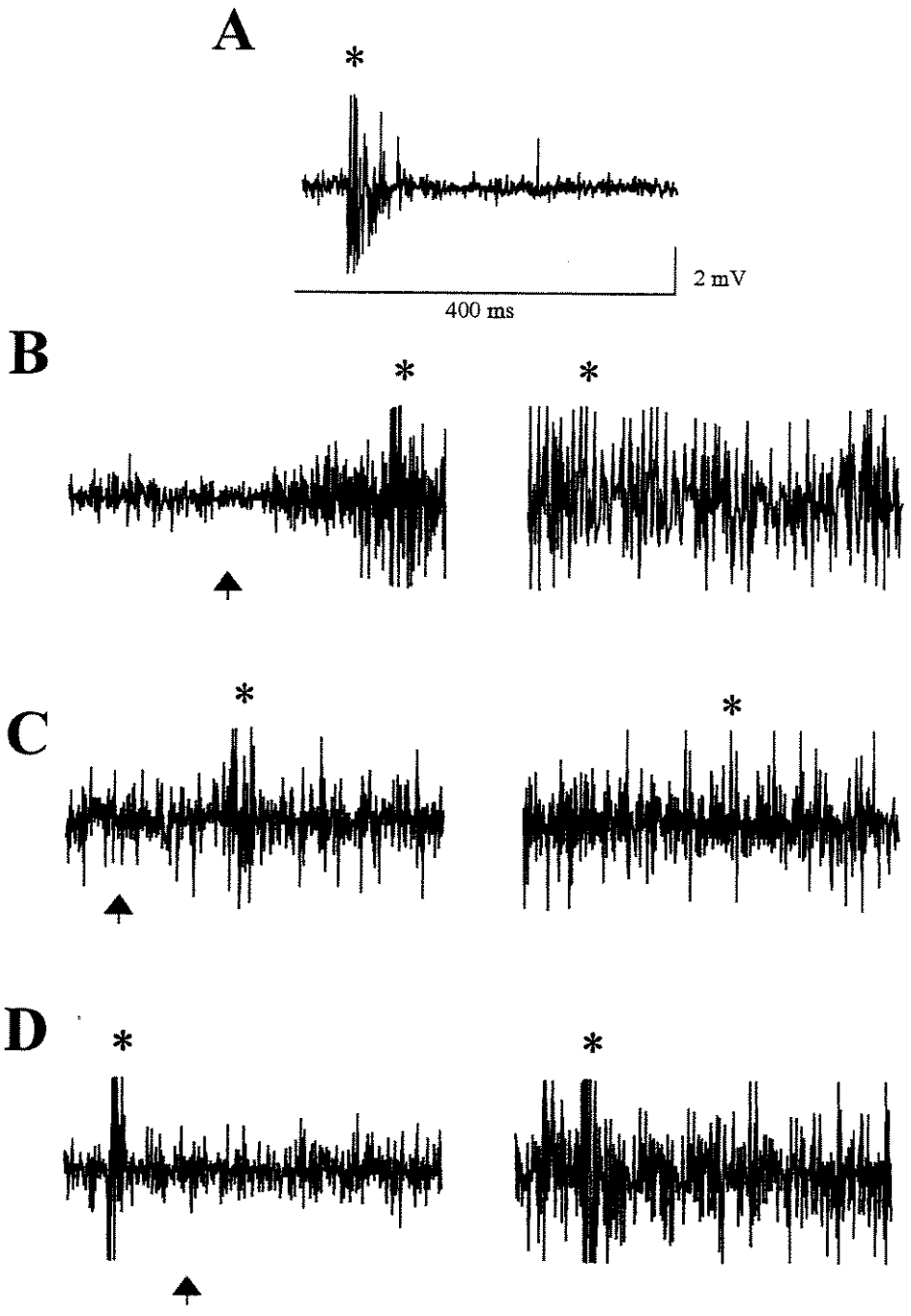


Figure 1

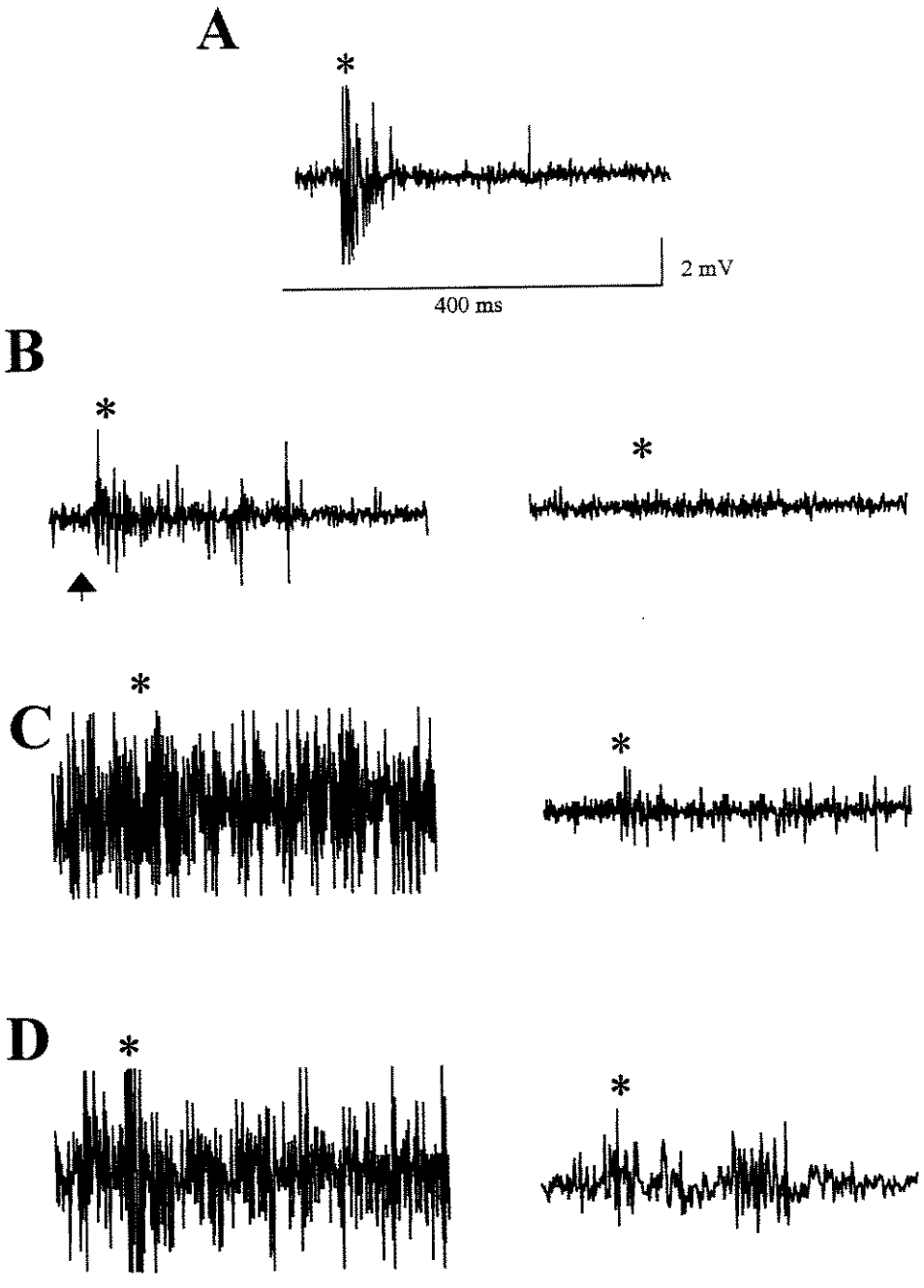
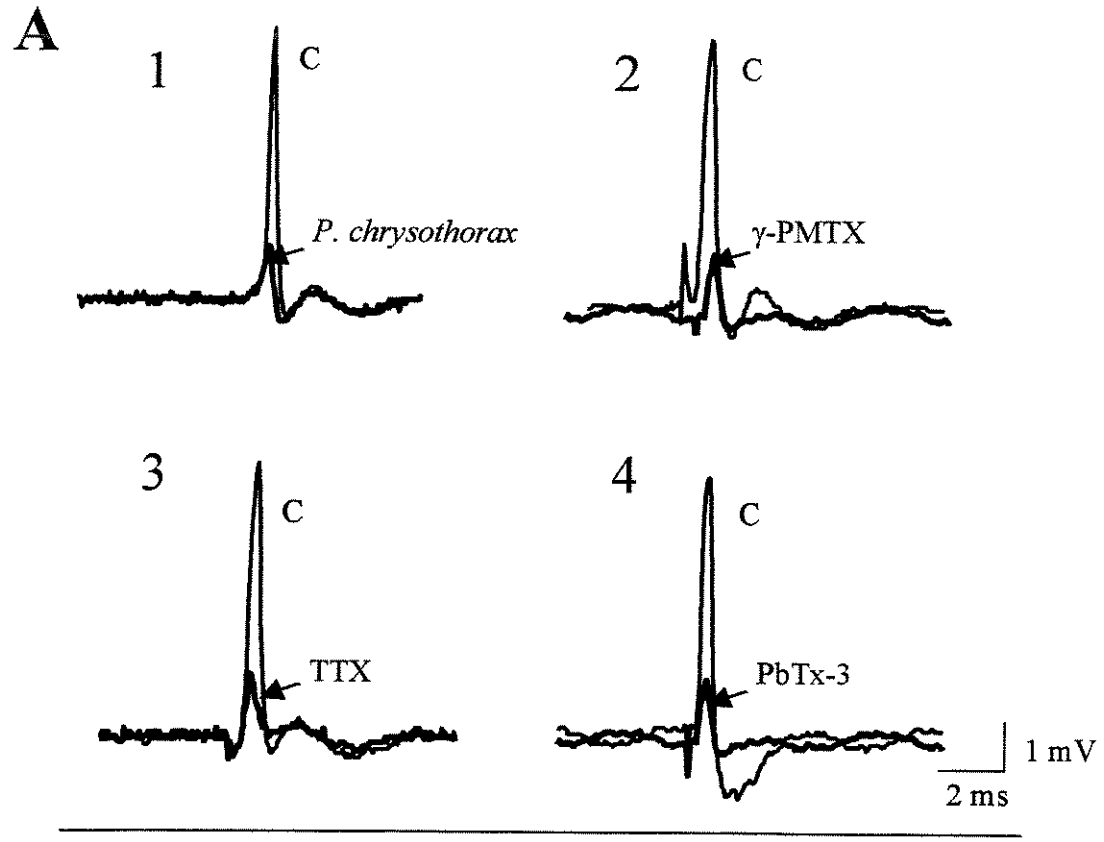


Figure 2


A
1
C
P. chrysothorax
2
C
γ-PMTX
3
C
TTX
4
C
PbTx-3
1 mV
2 ms

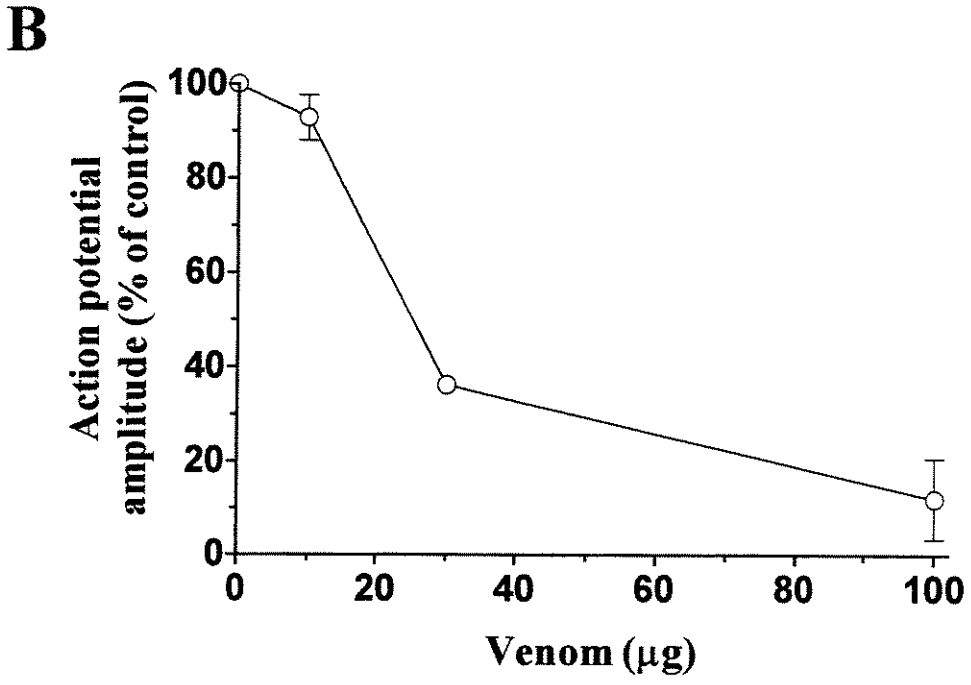

B
Action potential amplitude (% of control)
100
80
60
40
20
0
0
20
40
60
80
100
Venom (μg)

Figure 3

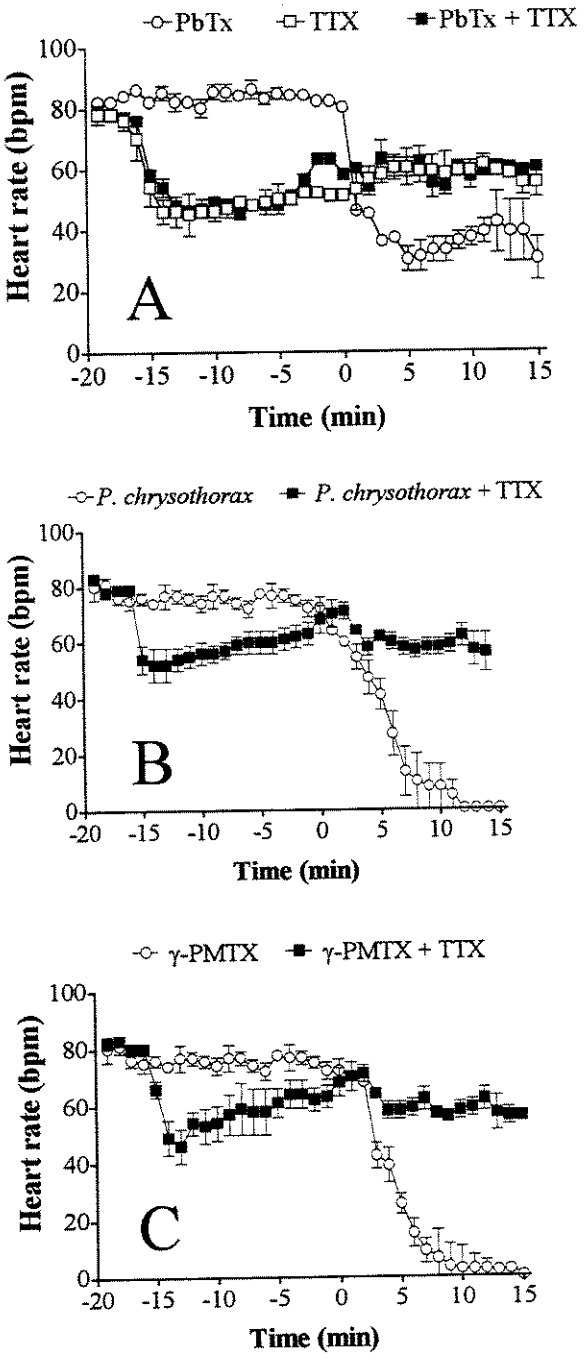
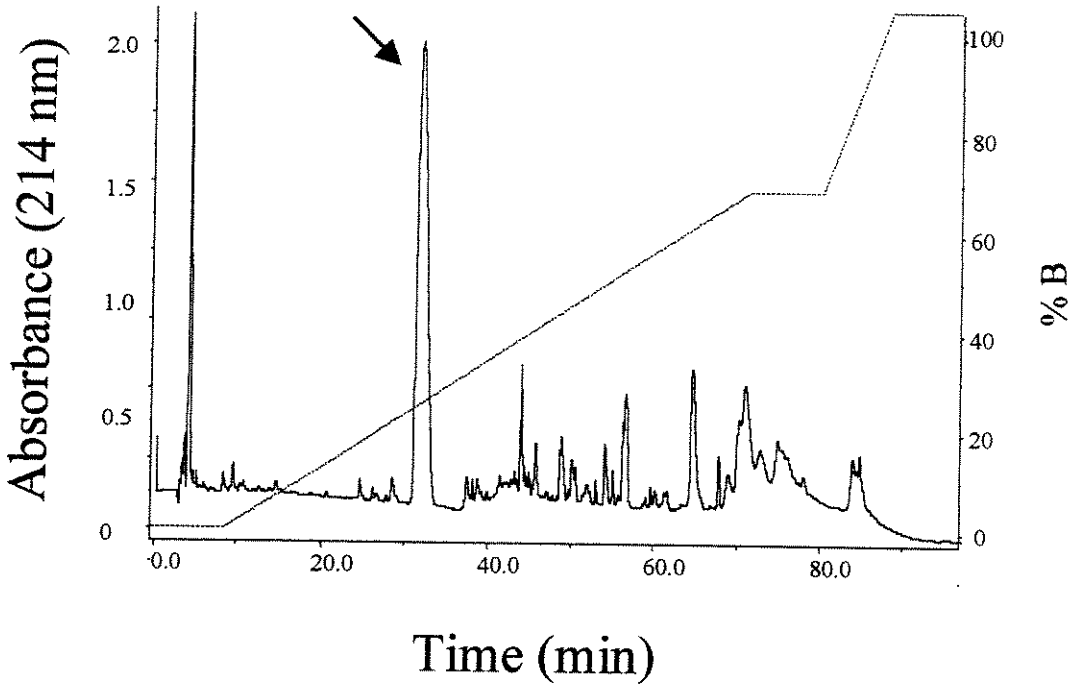

PbTx
TTX
PbTx + TTX
Heart rate (bpm)
A
Time (min)
P. chrysothorax
P. chrysothorax + TTX
Heart rate (bpm)
B
Time (min)
γ-PMTX
γ-PMTX + TTX
Heart rate (bpm)
C
Time (min)

Figure 4



	1	2	3	4	5	6	7	8	9	10	11	12	13				
γ-PMTX	A	R	**S**	**L**	L	E	G	**L**	**G**	**I**	R	_	**R**	G	S	A	
α-PMTX	L	K	**S**	**L**	Q	D	F	**L**	**G**	**I**	K	I	**R**				
β-PMTX	L	R	**S**	**L**	Q	D	F	**L**	**G**	**I**	K	I	**R**				

Figure 5

4

Neuromuscular action of venoms from some Brazilian social wasps in mouse phrenic nerve-diaphragm muscle preparations

NEUROMUSCULAR ACTION OF VENOMS FROM SOME BRAZILIAN SOCIAL WASPS IN MOUSE PHRENIC NERVE-DIAPHRAGM MUSCLE PREPARATIONS

Ericleison Cardoso-Silva[1], Chàriston André Dal Belo[1], Gildo Bernardo Leite[1], Léa Rodrigues-Simioni[1], Gustavo Henrique da Silva[2], Maria Alice da Cruz-Höfling[2], Stephen Hyslop[1,*]

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

[2]Departamento de Histologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, 13083-970, Campinas, SP, Brasil

Running title: Wasp venom and neurotransmission

***Author for correspondence:** Dr. Stephen Hyslop, Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), CP 6111, 1308-970, Campinas, SP, Brasil. Tel. (55)-(19)-3788-9536. Fax: (55)-(19)-3289-2968. E-mail: hyslop@fcm.unicamp.br

Abstract

The neuromuscular action of venoms from three species of social wasps (*Polybia chrysothorax, Polybia rejecta* and *Pseudopolybia compressa*) from northeastern Brazil were examined in mouse phrenic nerve-diaphragm preparations. The venoms of *P. chrysothorax* and *P. compressa* (25, 50 and 100 μg/ml) produced concentration- and time-dependent irreversible (by washing), neuromuscular inhibition of muscle twitch responses, with complete inhibition occurring after 120 min at the highest venom concentration (100 μg/ml). *P. rejecta* had little effect in these preparations. The venoms of *P. chrysothorax* and *P. compressa* also reduced the twitch responses in chronically denervated mouse diaphragm and markedly inhibited the contractions caused by acetylcholine (ACh). *P. chrysothorax venom* depolarized the muscle fiber membrane and enhanced the frequency of miniature end-plate potentials that had amplitudes of 0.3-1 mV. Histological analysis showed that the two most active venoms of *P. chrysothorax* and *P. compressa* caused marked myonecrosis characterized by membrane rupture, myofibril disintegration and vacuolation. These findings indicate that the venoms of the social wasps examined interfered with mammalian neurotransmission and caused morphological alterations to the muscle. The electrophysiological results obtained for *P. chrysothorax* suggest that this venom affects nicotinic receptors directly (as seen by the reduced reponses to ACh) but may also have a presynaptic action.

Key words: neuromuscular junction, neurotransmission, phospholipase A_2, phrenic nerve-diaphragm, Vespidae

1. Introduction

Wasp venoms contain a variety of components with neurotoxic activity in invertebrate and vertebrate preparations. These compounds include phospholipases A (Ho and Ko, 1988; Oliveira and Palma, 1998; Costa and Palma, 2000), kinins, which cause presynaptic blockade of cholinergic neurotransmission in insects (Piek, 1991), toxins such as α- and β-pompilidotoxins (α and β-PMTXs) from the venoms of the solitary wasps *Anoplius samariensis* and *Batozonellus maculifrons*, respectively, which delay the inactivation of voltage-gated sodium channels (Konno *et al.*, 1998, 2000; Sahara *et al.*, 2000), and polyamines, such as philanthotoxin (Piek, 1982; Kits and Piek, 1986), which act principally at glutamate receptors in insect neurons (Eldefrawi *et al.*, 1993). Compared to their effects on invertebrate preparations, the actions of wasp venoms on neurotransmission in mammals is not well known. Hornetin, an 18 kDa toxin isolated from the venom of *Vespa flavitarsus*, blocks neurotransmission in chick biventer cervicis and mouse phrenic nerve-diaphragm preparations (Ho and Ko, 1986). Since little is known of the neuromuscular actions of Brazilian hymenopteran venoms, we have examined the effects of venoms from three social wasp species found in northeastern Brazil on neurotransmission in mouse phrenic nerve-diaphragm preparations. The morphological damage produced by the venoms was also examined by histological analysis.

2. Materials and Methods

2.1. Reagents

Acetylcholine (ACh) was from Sigma Chemical Co (St. Louis, MO, EUA) and chloral hydrate was from Vtec Químicas Finas (Rio de Janeiro, RJ, Brazil). The salts for Tyrode solutions

were from J.T. Baker (Xalostoc, Mexico), Mallinckrodt (Paris, KY, USA) or Merck (Rio de Janeiro, RJ, Brazil).

2.2. Animals

Adult male Swiss white mice (18-22 g) were supplied by the Central Animal House Service at UNICAMP. The mice were housed at 24°C with access to food and water *ad libitum.*

2.3. Collection of venom

Colonies of the social wasps *Polybia chrysothorax, Polybia rejecta* and *Pseudopolybia compressa* were collected in the coastal forest zone of the state of Pernambuco in northeastern Brazil. The insects were killed with CO_2 and stored to -20°C until used. When required, the wasps were thawed on ice and the venom sacs removed along with the sting. Following removal of the sting, the venom was obtained by gently pressing (without squashing) the venom sacs in a beaker to expel their liquid content after which the sacs were stirred gently in distilled water with a magnetic stirrer at 4° C to ensure maximum recovery of venom. The venom sacs were subsequently removed by centrifugation (4200 rpm, 10 min) and the supernatant then recovered, lyophilized and stored to -20° C until required. The protein content of the material thus obtained was determined by the method of Lowry as modified by Peterson (1977), using bovine serum albumin as a standard. All amounts of venom mentioned below refer to this protein content.

2.4. Phrenic nerve-diaphragm muscle preparation

The phrenic nerve and diaphragm were obtained from mice amd mounted essentially as described by Bülbring for rats (1946). The mice were anesthetized with chloral hydrate (300

mg/kg, i.p.) and exsanguinated by sectioning the neck vessels. The preparation was removed and mounted under a resting tension of 5 g in an organ bath containing 3.5 ml of warm (37 °C) Tyrode solution (composition, in 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, pH 7.4) which was gassed continuously with 95%O_2/5%CO_2. The preparation was stimulated indirectly through the phrenic nerve (Grass S48 stimulator) with supramaximal pulses of 0.2 ms duration at a frequency of 0.1 Hz. The muscle twitches were registered on a Gould RS 3400 recorder via an isometric transducer (Load Cell BG-10 GM). After allowing 15 min for the preparation to stabilize, venom was added to the bath and the contractile responses then recorded for up to 120 min.

2.5. Chronically denervated diaphragm

Mouse diaphragms were chronically denervated as described by Vital Brazil (1965) and used 15 days later. Hemidiaphragm preparations obtained essentially as described in Section 2.4 were mounted under a resting tension of 5 g and connected to a Gould RS 3400 recorder via an isometric transducer (Load Cell BG-10 GM). The preparations were stimulated directly with supramaximal stimuli (80 V, 2 ms, 0.1 Hz) delivered by a Grass S48 stimulator. After approximately 15 min for stabilization, contractile responses to acetylcholine (ACh, 50 and 100 μM) were obtained before the addition of *P. chrysothorax* venom (100 μg/ml) and at the end of the experiment. Twitch-tension responses were recorded for up to 120 min after the addition of venom.

2.6. Electrophysiological studies

Miniature end-plate potentials (MEPPs) and resting membrane potentials (RP) were measured in mouse phrenic nerve-diaphragm preparations using conventional microelectrode techniques. The dissected muscle was mounted in a lucite chamber containing aerated (95%O_2-5%O_2) Tyrode solution (pH 7.4, 23-27°C) with or without *P. chrysothorax* venom (100 μg/ml). Intracellular microelectrodes filled with 3 M KCl (resistance 15-25 MΩ) were coupled to a Bimos operational amplifier (model CA 3140) with a typical input impedance of 1.5 TΩ or to an electrometer of high input impedance (World Precision, 750). Microelectrode placement was considered adequate when the maximum amplitude of the MEPPs was reached in less than 1 ms. The MEPPs and muscle RP were recorded on a Tektronix oscilloscope and, in the case of MEPPs, were magnified (AM 502 Tektronix amplifier, gain = 100), low-pass filtered (3 kHz) and digitized (15 kHz sampling rate) using an analog-to-digital converter (Lynx, CAD12/36, resolution: 12 bits) coupled to a microcomputer (Magitronic) loaded with software (AqDados 5, Lynx) that enabled digital storage of the MEPPs as well as their subsequent retreival for measurement and analysis. RP recordings were obtained at or distant from the end-plate region in the absence or presence of *P. chrysothorax* venom (100 μg/ml)

2.7. Histological analysis

The ability of the venoms to damage diaphragm muscle was assessed histologically in phrenic nerve-diaphragm preparations incubated with *P. chrysothorax* and *P. compressa* venom in Tyrode solution (composition in Section 2.3) at 37 °C for up to 120 min. At the end of this period, the muscles were fixed in Bouin solution for 24 h, dehydrated in an increasing ethanol series and then embedded in paraffin (Histosec Pastilles Pac, Merck KGaA- Germany). Sections 3 μm thick

were cut with a Bio-Rad JB-4 histomicrotome and stained with hematoxylin-eosin. The extent of muscle damage was determined by calculating the percentage of damaged fibers relative to the total number of fibrescounted. Control preparations were incubated with Tyrode solution alone and processed as described above.

2.8. Statistical analysis

The results were expressed as the mean ± SEM as appropriate and were compared statistically using Student´s unpaired *t*-test (electrophysiological studies) or ANOVA for repeated measures (myographic studies). A value of $p < 0.05$ indicated significance.

3. Results

3.1. Inhibition of neuromuscular transmission by wasp venoms

The venoms of *P. chrysothorax* and *P. compressa* (25, 50 and 100 μg/ml) produced progressive inhibition of neuromuscular transmission in indirectly stimulated mouse phrenic nerve-diaphragm preparations, with complete inhibition occurring after 120 min at the highest concentration of *P. chrysothorax* venom (Fig. 1). This inhibition was not reverted by washing the preparations. *P. chrysothorax* venom (100 μg/ml) produced 50% inhibition in 40±5 min (n=3), while *P. compressa* venom (100 μg/ml) caused a similar level of inhibition in 57±3 min (n=3). In contrast, *P. rejecta* venom had little effect on the contractile responses of diaphragm muscle (25% reduction in the contractile responses after a 120 min incubation with the highest concentration of venom – 100 μg/ml; results not shown).

3.2. Effect of wasp venoms on chronically denervated diaphragm muscle

The effects of *P. chrysothorax* and *P. compressa* venoms on chronically denervated diaphragm muscle under direct electrical stimulation are shown in Figure 2. *P. chrysothorax* venom (100 μg/ml) abolished the contractile responses to ACh (50 and 100 μM) after 120 min, and also caused a decrease in the muscle twitch-tension responses (Fig. 2). *P. compressa* venom (100 μg/ml) had a similar effect on the twitch-tension responses but did not compeletely abolish the responses to ACh. The inhibitory effects of both venoms on the muscle twitches and responses to ACh were not reversed by washing the preparations.

3.3. Effect of P. chrysothorax venom on the membrane resting potential

P. chrysothorax venom (100 μg/ml) depolarized muscle fibers and decreased the membrane resting potential at and distant from the motor end-plate region after 120 min (-80 ± 0.4 mV and -75 ± 0.35 mV before and −18 ± 0.9 mV and -23 ± 0.8 mV 120 min after venom, respectively; $p<0.05$ compared to before venom; n=3 each) (Fig. 3).

3.4. Effect of P. chrysothorax venom on the frequency of mepps

The effect of *P. chrysothorax* venom (100 μg/ml) on the MEPPs frequency depended on the amplitude class and time interval examined. Thus, the venom markedly increased the frequency of 0.3-0.4 mV MEPPs from 90 min onwards while for 0.5-0.6 mV MEPPs, an initial decrease in amplitude was observed followed by an increase from 60 min onwards. For 0.7-1 mV MEPPs, there were increases after 10 min and 60 min, with decreases in the other intervals. The venom had no marked effect on the frequency of MEPPs greater than 1 mV. Giant MEPPs (2-3 mV) were observed throughout the experiments but were unaffected by the venom (n=3).

3.5. Morphological changes in diaphragm muscle

Morphological analysis of diaphragm muscle showed extensive damage to muscle fibers following incubation with the venoms of *P. chrysothorax* and *P. compressa* (36±1.2% and 64±0.8% of damaged fibers at 50 µg/ml and 100 µg/ml, respectively) (Fig. 5). The damage consisted mainly of membrane rupture fiber disintegration and extensive vacuolation.

4. Discussion

The results of this study show that the venoms of *P. chrysothorax* and *P. compressa* interfered with transmission at mammalian neuromuscular junctions in a concentration- and time-dependent manner. These results were similar to those reported for the venom of another Brazilian social wasp, *Polybia paulista,* which also caused concentration-dependent (10-200 µg/ml) blockade in this same preparation (Oliveira and Palma, 1998). These findings also agree with those of Edery *et al.* (1972) who reported that the venom of the oriental wasp, *Vespa orientalis,* blocked mammalian neuromuscular transmission in cat anterior tibialis, gastrocnemius and soleus muscles. Similarly, hornetin, a toxin from *Vespa flavitarssus* venom, blocks neurotransmission in chick biventer cervicis and mouse phrenic nerve-diaphragm preparations, probably via a presynaptic action (Ho and Ko, 1986). In addition, the neuromuscular effects of the wasp venoms examined here occurred in a concentration range similar to that seen with snake venoms of the genus *Bothrops* (Prianti *et al.* (2003) and refs therein).

The attentuated responses to acetycholine seen in chronically denervated diaphragms following exposure to venoms suggests that part of the action of these venoms was mediated by non-specific blockade or destruction of nicotinic receptors. However, this post-synaptic action was much less potent than that seen with snake venoms containing true blockers of post-junctional

nicotinic receptors (Goularte *et al.*, 1995; Tsai *et al.*, 1995). Similar findings have also been reported for venom from several *Bothrops* snake species (Rodrigues-Simioni *et al.*, 1983; Cogo *et al.*, 1993; Harvey *et al.*, 1994; Prianti *et al.*, 2003).

The electrophysiological results obtained with *P. chrysothorax* venom indicate muscle fiber membrane depolarization (changes in resting potential) and a presynaptic action involving nerve depolarization and an augmented release of neurotransmitter (changes in MEPPs frequencies). These findings could partly explain the attenuated response to exogenous acetylcholine in denervated preparations. The venoms of the spiders *Phoneutria nigriventer* (Fontana and Vital Brazil, 1985) and *Ancylometes* sp. (Grégio *et al.*, 1999) depolarize the muscle fibers in phrenic nerve-diaphragm preparations and increase the frequency of MEPPs, a finding compatible with the involvement of sodium channels in the inhibition of neurotransmission (see Vital Brazil and Fontana, 1993). These observations suggest that *P. chrysothorax* venom may contain toxins that can affect voltage-gated sodium channels and nicotinic acetylcholine receptors.

A direct action of the venoms on muscle fibers was also suggested by the moderate blockade of the muscle contractile responses seen in denervated preparations and by the extensive myonecrosis observed histologically. The myonecrosis produced by these wasp venoms was similar to that seen with their content of various *Bothrops* venoms, for which such damage has been associated with PLA_2 myotoxins present in the venoms (Gutiérrez and Lomonte, 1995). As reported elsewhere (Cardoso-Silva *et al.*, submitted), the three wasp venoms contained PLA_2 activity, with the order of potency being *P. chrysothorax* > *P. compressa* > *P. rejecta*. Interestingly, the extent of myonecrosis caused by the three venoms in diaphragm muscle generally agreed with the order of PLA_2, suggesting perhaps that this enzyme was involved in the effects observed.

In conclusion, the venoms of the social wasps studied here generally caused progressive, concentration-dependent inhibition of neurotransmission in mouse phrenic nerve-diaphragm preparations. This effect apparently involved postsynaptic and, to a lesser extent, presynaptic actions. The venoms also produced extensive myonecrosis which could indicate a direct toxic action on muscle fibers. The clinical relevance of these findings remains to be established.

Acknowledgments

The authors thank José Ilton dos Santos and Gilbo Bernardo Leite for technical assistance and Dr. Oton Meira Marques (Escola Politécnica de Agronomia, UFBA, Salvador, Bahia, Brazil) for identifying the wasps. E.C.S. and C.A.D.B. were supported by studentships from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant no. 00/05976-6). L.R.S., M.A.C.H. and S.H. are supported by research fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Bülbring E (1946) Observations on the isolated phrenic nerve-diaphragm preparation of the rat. *Br. J. Pharmacol.* **1,** 38-61.

Cogo JC, Prado-Franceschi J, Cruz-Höfling MA, Corrado AP, Rodrigues-Simioni L (1993) Effects of *Bothrops insularis* venom on the mouse and chick nerve-muscle preparation. *Toxicon* **31**, 1237-1247.

Costa H, Palma MS (2000) Agelotoxin: a phospholipase A_2 from the venom of the neotropical social wasp cassununga (*Agelaia pallipes pallipes*) (Hymenoptera – Vespidae). *Toxicon* **38**, 1367-1379.

Edery H, Ishay J, Gitter S (1972) Pharmacological activity of oriental hornet (*Vespa orientalis*) venom. *Toxicon* **10,** 13-23.

Eldefrawi ME, Anis NA, Eldefrawi AT (1993) Glutamate receptor inhibitors as potential insecticides. *Arch. Insect Biochem. Physiol.* **22,** 25-39.

Fontana MD, Vital Brazil O (1985) Mode of action of *Phoneutria nigriventer* spider venom at the isolated phrenic nerve-diaphragm of the rat. *Braz. J. Med. Biol. Res.* **18**, 557-565.

Goularte FC, Cruz-Höfling MA, Cogo JC, Gutiérrez JM, Rodrigues-Simioni L (1995) The ability of specific antivenom and low temperature to inhibit the myotoxicity and neuromuscular block induced by *Micrurus nigrocintus* venom. *Toxicon* **33**, 679-689.

Grégio AMT, Heleno MG, von Eicksted VR, Fontana MD (1999) The neuromuscular action of *Ancylometes* sp. spider venom in rat phrenic nerve-diaphragm preparation. *Toxicon* **37**, 545-550.

Gutiérrez JM, Lomonte B (1995) Phospholiase A^2 myotoxins from Bothrops sanke venoms. *Toxicon* **33**, 1405-1424.

Harvey AL, Barfarz A, Thompson E, Faiz A, Preston S, Harris JB (1994) Screening of snake venom for neurotoxic and myotoxic effects using simple *in vitro* preparations from rodents and chicks. *Toxicon* **32**, 257-265.

Ho CL, Ko JL (1986) Hornetin: the lethal protein of the hornet (*Vespa flavitarsus*) venom. *FEBS Lett.* **209**, 18-22.

Ho CL, Ko JL (1988) Purification and characterization of a lethal protein with phospholipase A_1 activity from the hornet (*Vespa basalis)* venom. *Biochim. Biophys. Acta* **963**, 414-422.

Kits KS, Piek T (1986) Action of the polyamine β-philanthotoxin on neuromuscular transmission in insects. *Neuropharmacology* **25,** 1089-1093.

Konno K, Hisada M, Itagaki Y, Naoki H, Nobufumi K, Miwa A, Yasuhara T, Takayama H (1998) Isolation and structure of pompilidotoxins, novel peptide neurotoxins in solitary wasp venoms. *Biochem. Biophys. Res. Commun.* **250,** 612-616.

Konno K, Hisada M, Naoki H, Itagaki Y, Yasuhara T, Nakata Y, Miwa A, Kawai N (2000) Molecular determinants of binding of a wasp toxin (PMTXs) and its analogs in the Na^+ channels proteins. *Neurosci. Lett.* **285,** 29-32.

Oliveira MR, Palma MS (1998) Polybiatoxins: a group of phospholipases A_2 from the venom of the neotropical social wasp paulistinha (*Polybia paulista*). *Toxicon* **36**, 189-199.

Peterson GL (1977) A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**, 357-363.

Piek T (1991) Neurotoxic kinins from wasp and ant venoms. *Toxicon* **29,** 139-149.

Piek T (1982) δ-Philanthotoxin, a semi-irreversible blocker of ion channels. *Comp. Biochem. Physiol.* **72C,** 311-315.

Prianti Jr ACG, Ribeiro W, Lopes-Martins RAB, Lira-Da-Silva RM, Prado-Franceschi J, Rodrigues-Simioni L, Cruz-Höfling MA, Leite GB, Hyslop S, Cogo JC (2003) Effect of *Bothrops leucurus* venom in chick biventer cervicis preparations. *Toxicon* (in press).

Rodrigues-Simioni L, Borgese N, Ceccarelli B (1983) The effects of *Bothops jararacussu* venom and its components on frog nerve muscle preparation. *Neuroscience* **10**, 475-489.

Sahara Y, Gtoh M, Konno K, Miwa A,Tsubokawa H, Robinson-Hugh PC, Kawai N (2000) A new class of neurotoxin from wasp venom the slows inactivation of sodium current. *Eur. J. Neurosci.* **12**, 1961-1970.

Tsai MC, Hsieh WH, Smith LA, Lee CY (1995) Effects of waglerin-I on neuromuscular transmission of mouse nerve-muscle preparations. *Toxicon* **33**, 363-371.

Vital Brazil O (1965) Ação neuromuscular da peçonha de *Micrurus*. *O Hospital* V **68**, 990-950.

Vital Brazil O, Fontana MD (1993) Toxins as tools in the study of sodium channel distribution in the muscle fibre membrane. *Toxicon* **31**, 1085-1098.

FIGURE LEGENDS

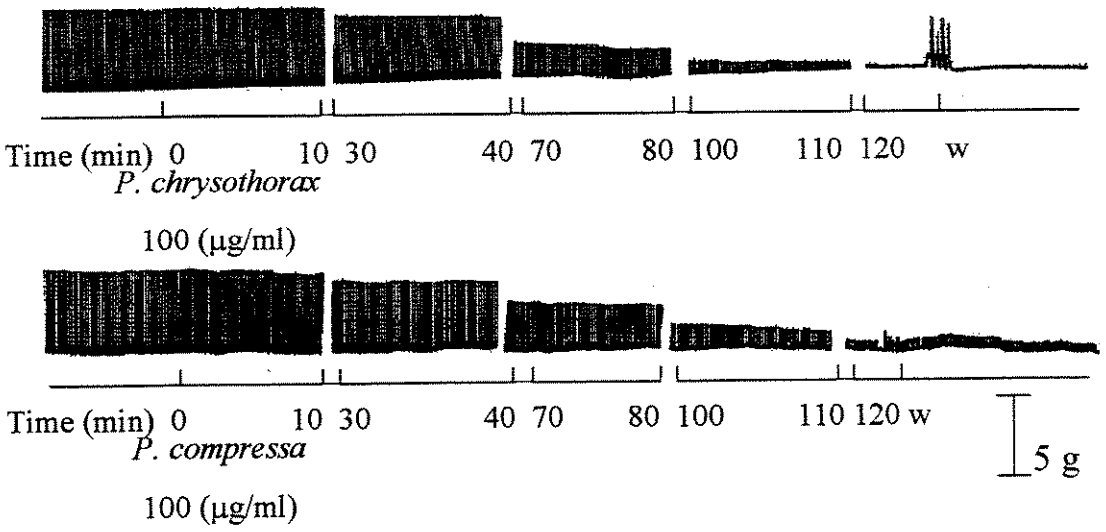
Figure 1. Inhibition by social wasp venoms of neurotransmission in mouse phrenic nerve-diaphragm preparations. The preparations were mounted and stimulated indirectly as described in Section 2. The recordings are from a representative experiment for each venom and the graphs show the mean responses (± SEM) for 3-4 experiments. W – wash.

Figure 2. Effect of social wasp venoms on muscle twitch response in chronically denervated mouse diaphragm. Note that the venoms moderately inhibited the responses to direct stimulation and also greatly reduced or nearly abolished the contractures caused by acetylcholine. The preparations were denervated and mounted as described in Section 2. The recordings are representative of three experiments.

Figure 3. Changes in the membrane potential of mouse diaphragm muscle caused by *P. chrysothorax* venom (100 μg/ml) at and distant from the motor endplate. The preparations were mounted as described in Section 2. The points are the mean ± SEM of four experiments.

Figure 4. Alterations in the frequency of miniature end-plate potentials (MEPPs) in mouse isolated diaphragm in response to *P. chrysothorax* venom (100 μg/ml). The preparations were mounted as described in Section 2. The columns are the mean ± SEM of 3-4 experiments.

Figure 5. Myonecrosis caused in mouse phrenic nerve-diaphragm preparations incubated with *P. chrysothorax* and *P. compressa* venom (100 μg/ml) for 120 min. HE staining. Magnification – 150x.


Time (min) 0 10 30 40 70 80 100 110 120 w
P. chrysothorax
100 (μg/ml)
Time (min) 0 10 30 40 70 80 100 110 120 w
5 g
P. compressa
100 (μg/ml)

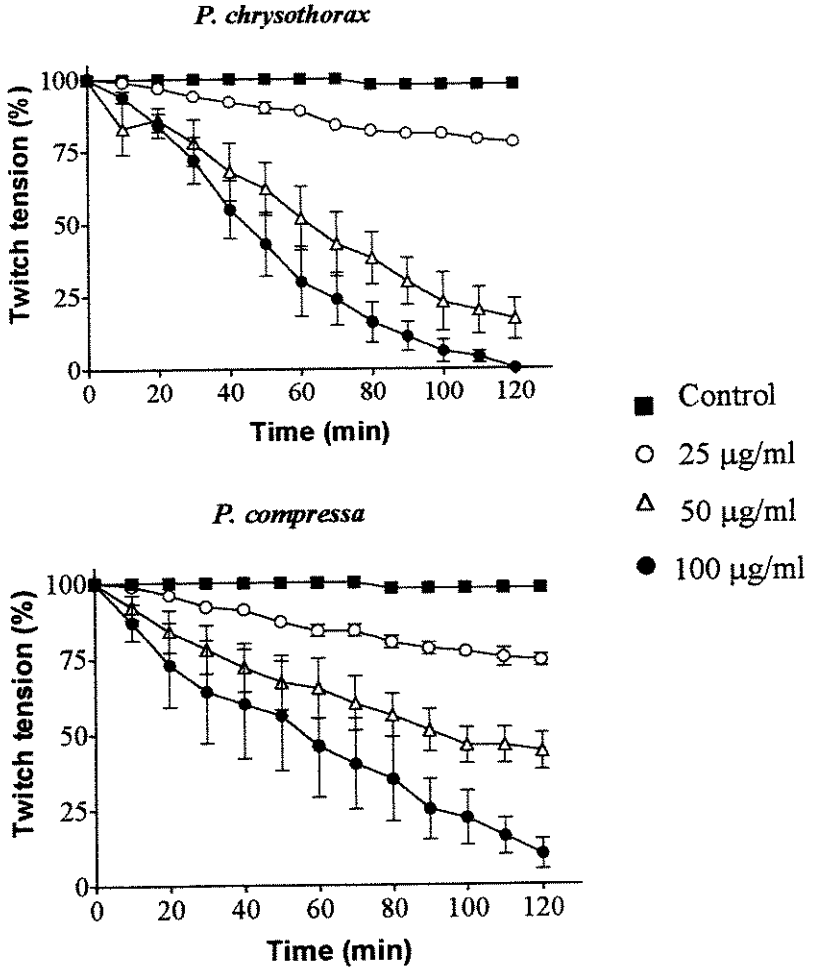

P. chrysothorax
Twitch tension (%)
Time (min)
P. compressa
Twitch tension (%)
Time (min)
Control
25 μg/ml
50 μg/ml
100 μg/ml

Figure 1

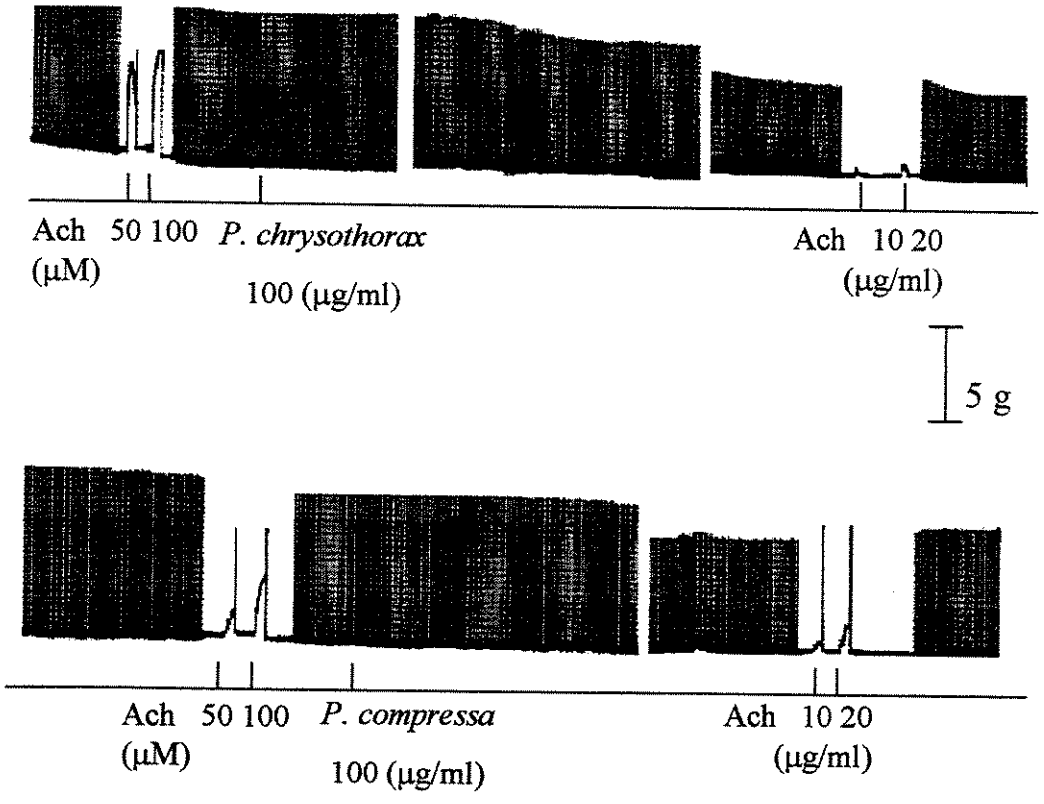

Ach 50 100 P. chrysothorax
(μM)
100 (μg/ml)
Ach 10 20
(μg/ml)
5 g
Ach 50 100 P. compressa
(μM)
100 (μg/ml)
Ach 10 20
(μg/ml)

Figure 2

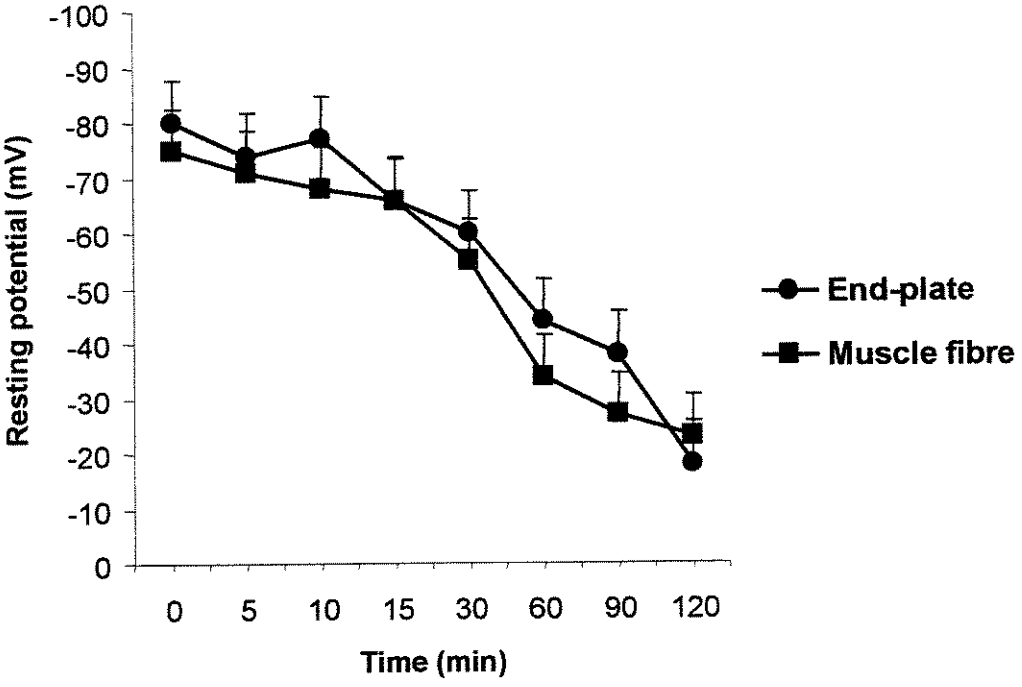

-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
0
Resting potential (mV)
0
5
10
15
30
60
90
120
Time (min)
End-plate
Muscle fibre

Figure 3

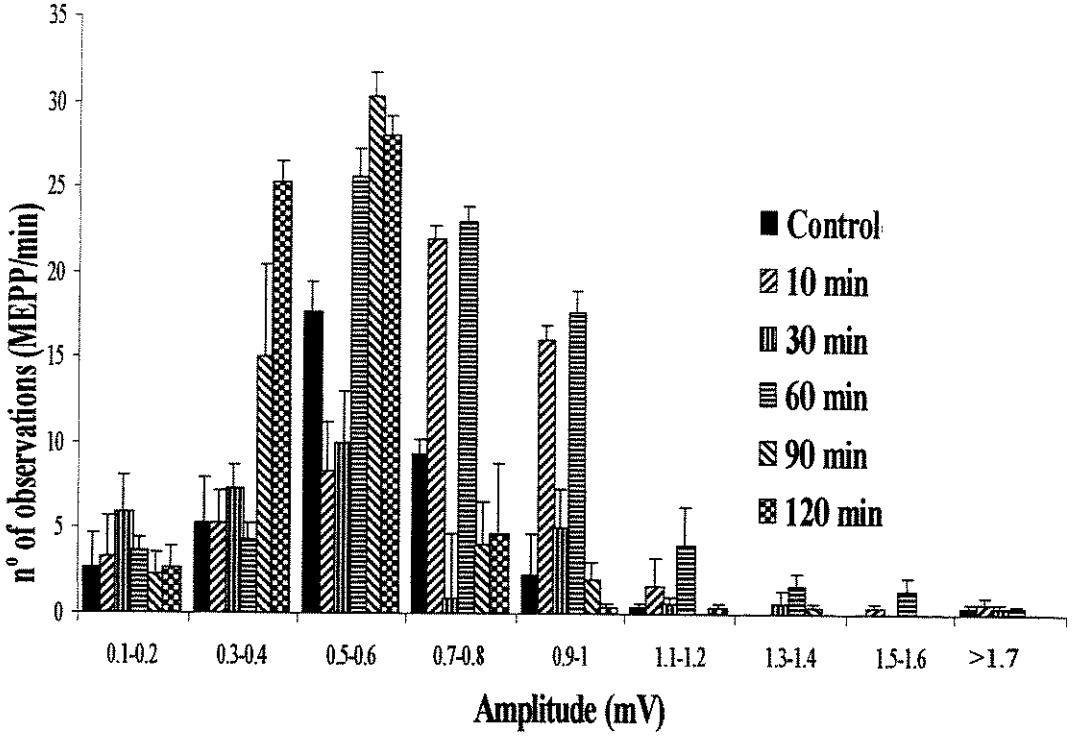

35
30
25
20
15
10
5
0
n° of observations (MEPP/min)
Control
10 min
30 min
60 min
90 min
120 min
0.1-0.2
0.3-0.4
0.5-0.6
0.7-0.8
0.9-1
1.1-1.2
1.3-1.4
1.5-1.6
>1.7
Amplitude (mV)

Figure 4

Control

P. chrysothorax

P. compressa

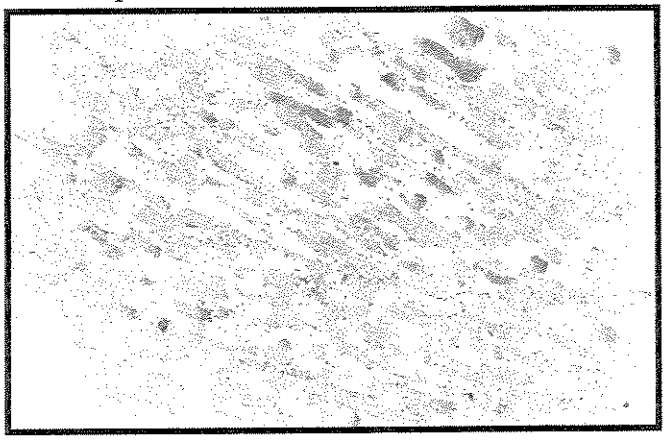


Figure 5

RESULTADOS ADICIONAIS

RESULTADOS ADICIONAIS

No artigo 3 deste trabalho foi utilizada preparações eletrofisiológicas de estimulação de nervo sensorial de crustáceos. Esta preparação consiste em avaliar os efeitos neurotóxicos de substâncias biologicamente ativas. Resumidamente,. a preparação foi feita com crustáceos decápodes braquiúros da espécie *Callinectes danae* (siri azul), adultos. O segundo ou terceiro pares de apêndices locomotores foram separados provocando autotomia por compressão do artículo proximal (base-ísquio). Os artículos foram removidos por secção das membranas articulares (artrodiais) e liberação das articulas de cada um, possibilitando sua remoção como uma luva de um dedo. O dáctilo com nervo apenso foi transportado para uma câmara de registro, montada em uma Gaiola de Faraday para eliminar as interferências externas. A extremidade do dáctilo permaneceu exposta afim de ser estimulado por gotas de água do mar filtrada que caia de uma altura de cerca de 15 cm a uma freqüência constante. O feixe nervoso passou por uma pequena câmara de 100 µl para aplicação do veneno testado e foi conectado a um eletrodo de sucção (Fig. 1). O nervo foi mantido em solução fisiológica para *Callinectes danae.* Os venenos foram diluídos em solução fisiológica e aplicados no compartimento na quantidade de 100 µg, que representa a dose de 100% de resposta.

Nas preparações de "sucrose gap", o nervo sensorial foi removido de acordo com o procedimento anterior, porém, seccionando-se o nervo, separando do dáctilo e isolando eletricamente através de lavagens sucessivas com solução de sucrose 1M em câmaras de acrílico (Fig. 2). Nesta preparação foram utilizadas as quantidades de 10, 30 e 100 µg, para a curva dose-resposta.

Os registro foram coletados através do programa de aquisição de dados Whole Cells Electrophysiology Program (WCP) versão 1.2 (Digidata 1200, Axon Instruments, Union City, CA, USA).

As figuras 3, 4 e 5 representam a purificação e testes biológicos realizados com a peçonha da vespa *P. compressa*, seguindo os protocolos descritos no artigo 3 (Pág. 99). Nas figuras 6 e 7, são mostradas as ações das peçonhas das vespas sociais *Polybia rejecta* e as espécies solitárias *Pypper* sp. 1 e *Pypper* sp. 2. Nas preparações de nervo frênico-diafragma de camundongos conforme descrito no artigo 4 (Pág. 131). De um modo geral estas vespas apresentaram pouca ou nenhuma atividade sobre as preparações utilizadas neste trabalho.

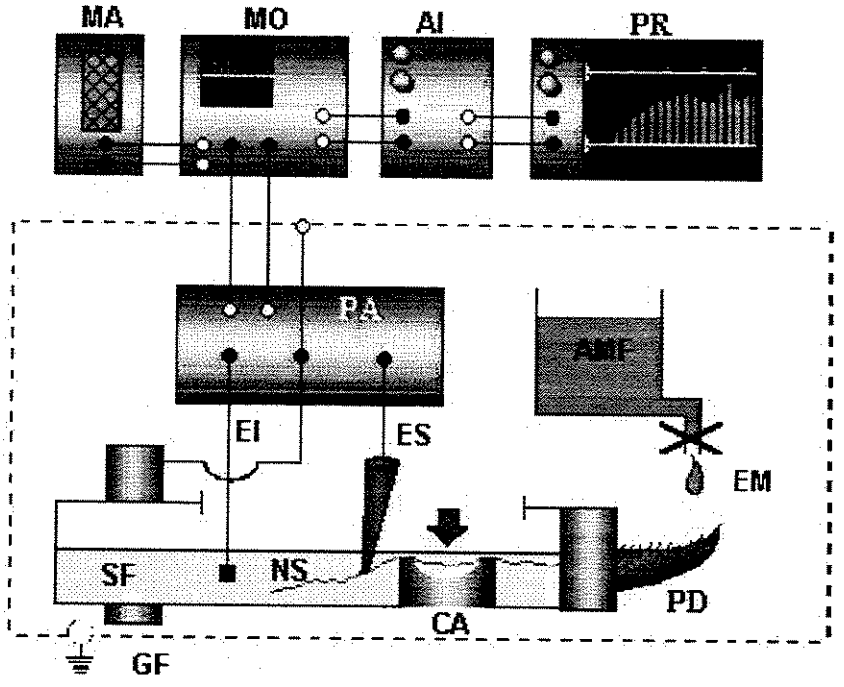


FIGURA 1. Diagrama de montagem eletrofisiológica da preparação de nervo sensorial isolado de *C. danae*. CA= câmara de aplicação, PD= dáctilo, NS= nervo sensorial, SF= solução fisiológica, EI= eletrodo indiferente, ES= eletrodo de sucção, AMF= água do mar filtrada, EM= estímulo mecânico, PA= pré-amplificador AC, MO= osciloscópio, MA= monitor de audio, AI= integrador, PR= polígrafo, GF= gaiola de Faraday.

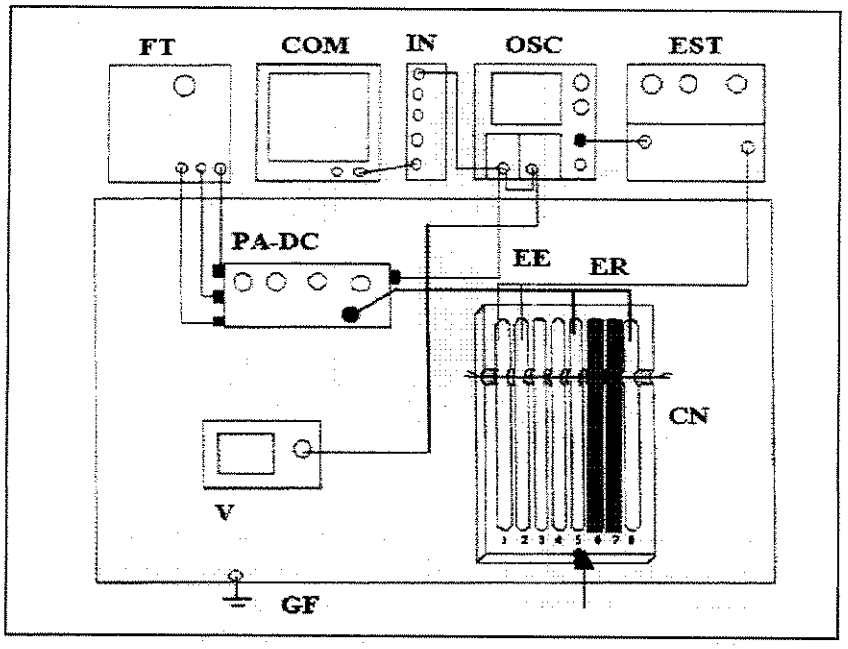


FIGURA 2. Esquema de montagem eletrofisiológica da preparação de nervo sensorial isolado de *C. danae* na técnica de "sucrose-gap". CN= câmara do nervo, EE= eletrodo de estímulo, ER= eletrodo de registro, PA-DC= pré-amplificador DC, V= voltímetro, OSC= osciloscópio, FT= fonte, COM= computador, IN= interface A/D, GF= gaiola de Faraday. A seta indica a câmara de 100 µl onde são aplicadas as amostras a serem testadas.

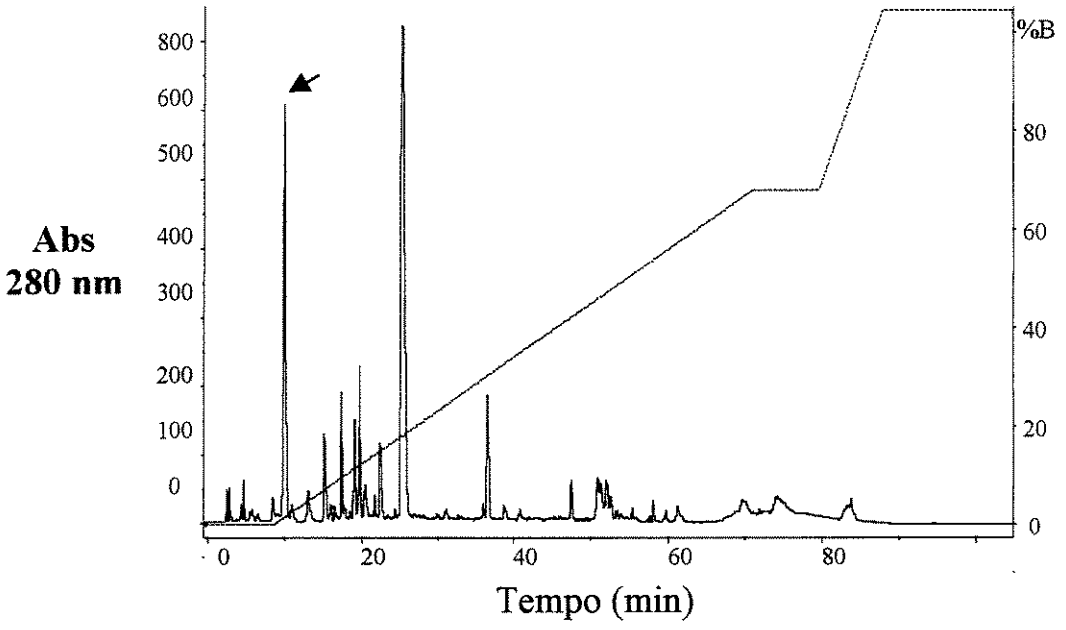


FIGURA 3. Perfil da cromatografia de fase reversa HPLC em coluna de Sephasil C18 da peçoonha de *P. compressa*. A coluna foi equilibrada com TFA 0,1% e as proteínas eluídas com gradiente 0-65% de acetonitrila 66%, com fluxo de 1 ml/min. Perfil de eluição foi monitorado a 280 nm. A seta indica a fração ativa

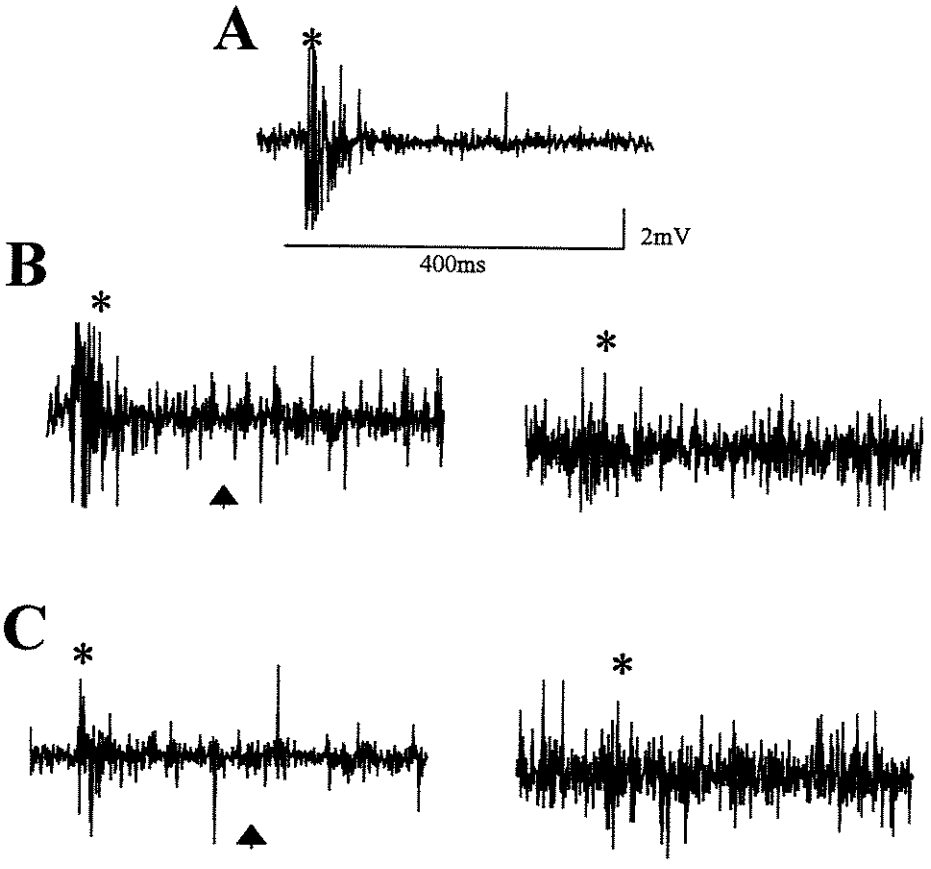


FIGURA 4. Nervo sensorial isolado em estimulo dos mecanorreceptores do dáctilo. Efeitos da peçonha (B)e fração (C) isolada da vespa *P. compressa* (100 µg). (A) registro controle dos potenciais deflagrados. Os símbolos (*) e seta representam os pontos de estimulação e de aplicação da peçonha ou fração, respectivamente. Os registros são representativos de 3 a 4 experimentos cada

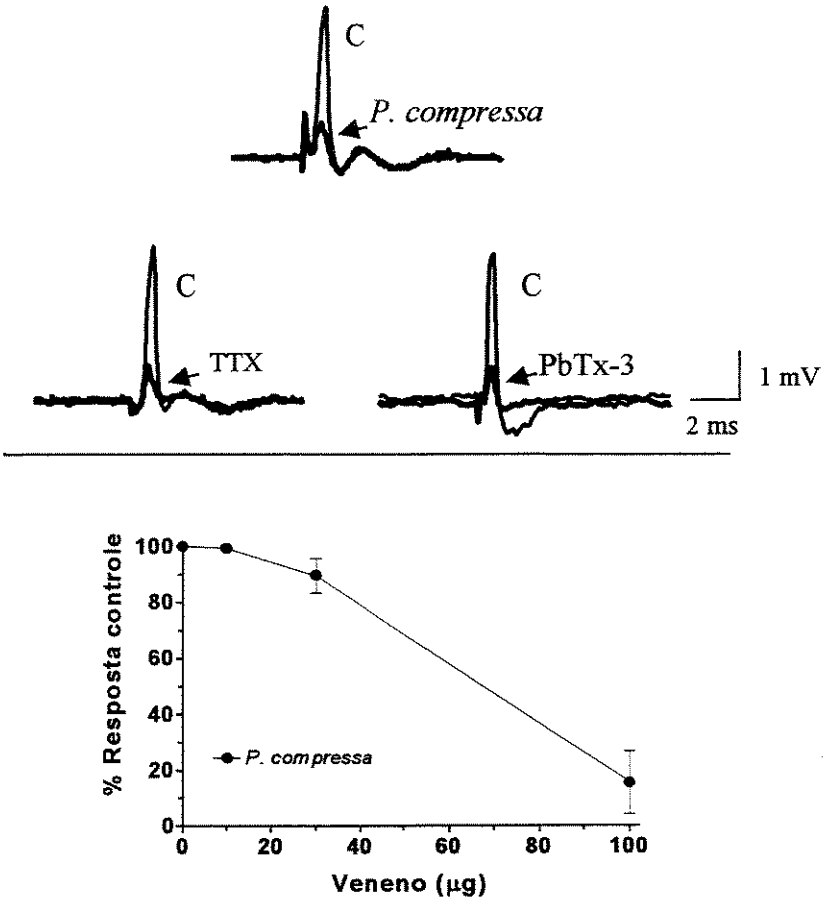


FIGURA 5. Nervo sensorial isolado em sucrose gap, com estímulos elétricos. Bloqueio do potencial de ação evocado na presença da peçonha (100 µg) de *P. compressa* (A). (B) Bloqueio causado pela TTX (10^{-6} M). (C) Inibição do potencial de ação por despolarização da fibra nervosa provocada pela PbTx-3 ($5x10^{-7}$ M). C = potenciais controles. O gráfico em baixo representa a cuva dose-resposta (10, 30 e 100 µg) para a peçonha *P. compressa*. Os registros são representativos de 3 a 4 experimentos cada

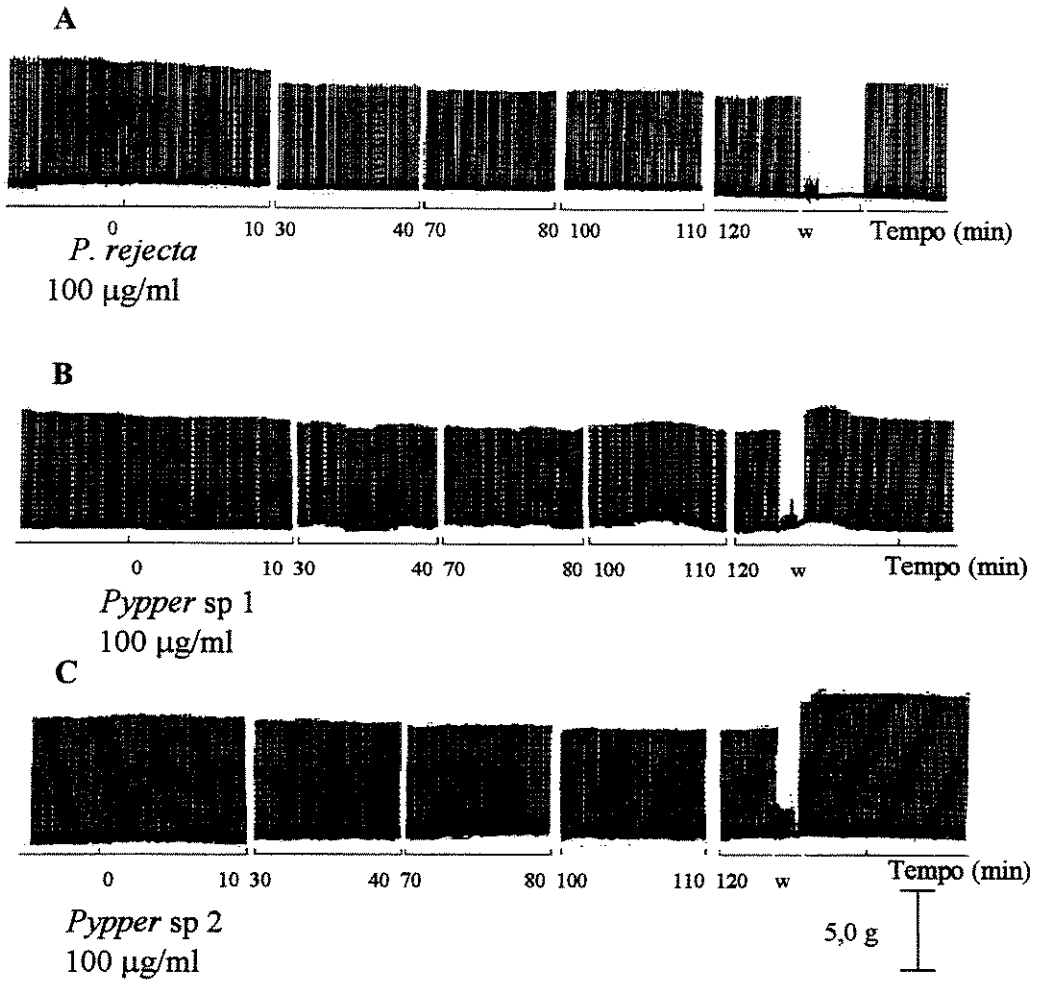


FIGURA 6. Ação de veneno de vespas sociais (*P. rejecta*) e solitárias (*Pypper* sp 1 e sp 2) sobre a neurotransmissão em preparações nervo frênico-diafragma de camundongos. Os registros são representativos das respostas de 3 preparações

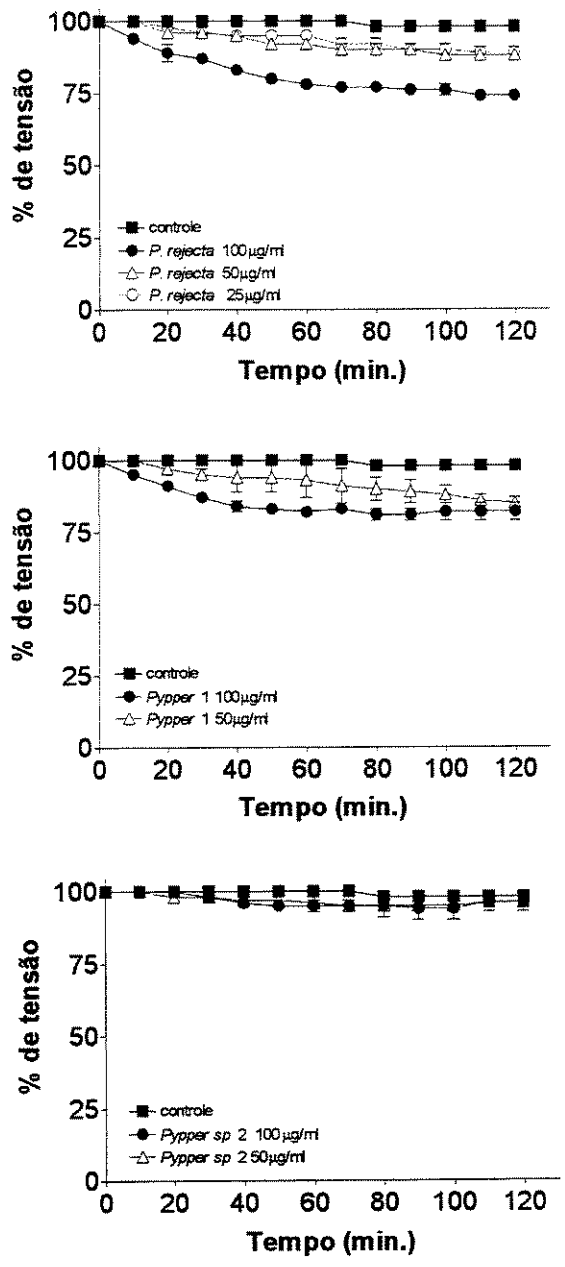


FIGURA 7. Ação de veneno de vespas sociais (*P. rejecta*) e solitárias (*Pypper* sp 1 e sp 2) sobre a neurotransmissão em preparações nervo frênico-diafragma de camundongos. Os pontos representam a média±EPM de 3 preparações

DISCUSSÃO GERAL

DISCUSSÃO GERAL

As peçonhas de vespas contêm uma variedade de componentes de baixo peso molecular, incluindo aminoácidos, aminas biogênicas e peptídeos (ver Introdução), e os resultados da presente série de estudos indicam que tais componentes são responsáveis pelos efeitos observados em nosso trabalho.

O artigo 1 (pág. 49) demonstra, através do uso de antagonistas seletivos para acetilcolina (atropina), histamina (pirilamina), 5-HT (metisergida) e bradicinina (Hoe-140), que as três peçonhas das vespas sociais contêm aminas biogênicas como constituintes e que estão desprovidas de cininas. No caso da *P. chrysothorax* e *P. rejecta*, os principais mediadores das respostas contráteis em íleo isolado de cobaia foram a histamina e serotonina enquanto que na peçonha de *P. compressa*, este mediador parece ser a acetilcolina. Estes resultados concordam com a presença destas substâncias em outros venenos de vespa. Assim, por exemplo, acetilcolina ocorre em grandes quantidades nas peçonhas das vespas sociais *Vespa crabro* (HABERMANN, 1972) e *Vespa orientalis* (EDERY *et al.*, 1972). Histamina foi identificada na peçonha das vespas *Megascolia flavifrons* (PIEK *et al.*, 1983) e *Vespa crabro* e *Vespa vulgaris* (EDERY *et al.*, 1978), enquanto que a serotonina foi detectada nas peçonhas de *V. orientalis*, *V. vulgaris* e *P. rejecta* (EDERY *et al.*, 1972, 1978). Já a peçonha da vespa solitária *Philanthus triangulum* é desprovida tanto de histamina como de serotonina (PIEK *et al.*, 1983).

As cininas são amplamente distribuídas em peçonhas de vespa, especialmente nas sociais (YOSHIDA *et al.*, 1976; HORI *et al.*, 1977; NAKAJIMA, 1984; YASUHARA *et al.*, 1987; PIEK, 1984, 1991), de modo que a ausência destes peptídeos nas espécies estudadas aqui é interessante. Em estudo anterior (BUZIN, 1999), observamos a presença de cininas na peçonha da

vespa social *Polistes lanio lanio*. Ao contrário das sociais, as vespas solitárias geralmente estão destituídas de cininas (KONNO *et al.*, 2002).

Partindo destes dados, as ações das peçonhas sobre preparações de invertebrados foram investigadas. Órgãos e tecidos isolados de artrópodes têm sido amplamente usados em investigações farmacológicas e fisiológicas para estudar a ação de substâncias endógenas e identificar toxinas isoladas de diversos venenos animais. Estes bioensaios têm favorecido a descoberta de numerosos compostos inseticidas e regulatórios (BROWN e STARRAT, 1975; MILLER, 1979; USHERWOOD e MACHILI, 1979; BAUMANN e GRECH, 1982; PASS *et al.*, 1988; CUTHBERT e EVANS, 1989; PREDEL *et al.*, 1994; LANGE *et al.*, 1995; AMAT *et al.*, 1997; EAST *et al.*, 1997; ECKER *et al.*, 1999; WEGENER e NÄSSEL, 2000; ROSE *et al.*, 2000; SLIWOWSKA *et al.*, 2001). Também têm sido empregadas na avaliação da toxicidade de venenos e toxinas de serpentes, escorpiões, aranhas e himenópteros (HORI *et al.*, 1977; ENTWISTLE *et al.*, 1982; PELHATES e ZOLTKIN, 1982; GAWADE, 1983; KITS e PIEKS, 1986, 1991; KLOWDEN *et al.*, 1992; BEN KALIFA *et al.*, 1997; KONNO *et al.*, 1998; SAUTIÈRE *et al.*, 1998; STANKIEWICZ *et al.*, 2000; FIGUEREDO *et al.*, 2001; HILL e BLAGBURN, 2001).

No artigo 2 (pág. 67), está demonstrado que não são as aminas biogênicas que exercem os efeitos tóxicos nas preparações de coração semi-isolado de barata (*Leucophea madere*) e sim toxinas que afetam principalmente os canais de sódio voltagem-dependentes. Outro fato interessante refere-se à presença de fosfolipase nestas peçonhas é que a depressão na freqüência cardíaca das baratas observada, não é mediada por estas fosfolipases, apesar da alta atividade enzimática, como apontam os resultados. Estas enzimas (FLA_1, FLA_2 e FLB) estão presentes em peçonhas de vespas (KING *et al.*, 1984; SCHMIDT *et al.*, 1986; HO e KO, 1988; TKASAKI e FUKUMOTO, 1989; VARGAS-VILLARREAL *et al.*, 1991; OLIVEIRA e PALMA, 1998;

COSTA e PALMA, 2000) e têm sido descritas como as responsáveis por produzirem contração em músculo liso, hemólise (OLIVEIRA e PALMA, 1998), degranulação de mastócitos (HABERMANN, 1972) e ação neurotóxica (EDERY *et al.*, 1972). Além de, juntas com a hialuronidase e antígeno 5, desencadearem as reações alérgicas após a picada (HOFMANN, 1985; LITTLER *et al.*, 1985; KING *et al.*, 1996).

Juntando as conclusões obtidas neste artigo 2 aos perfis cromatográficos das peçonhas mais ativas (ver artigo 3 e Resultados Adicionais), sugere-se que hajam toxinas ativas em canais de sódio e que representem os elementos de maior constituição nas peçonhas.

Na tentativa de responder a estas questões, recorreu-se à técnicas eletrofisiológicas. No artigo 3 (pág. 97) deste trabalho, preparações de nervo sensorial do crustáceo siri azul (*Callinectes danae*) (FREITAS *et al.*, 1992; MALPEZZI *et al.*, 1993) foram utilizadas (para descrição da aparelhagem, ver Resultados Adicionais). Os resultados obtidos mostram claramente que as peçonhas das vespas *P. chrysothorax* e *P. compressa*, assim como as respectivas frações ativas testadas, apresentaram como respostas um disparo de potenciais de ação independentes do estímulo mecânico. Semelhante efeito foi observado com a preparação na presença da PbTx-3, que despolariza as membranas excitáveis deixando o canal aberto, aumentando o influxo dos íons sódio e provocando salvas de potenciais de ação (RICHARDS *et al.*, 1990; SCHREIBMAYER e JEGLITSCH, 1992; JEGLITSCH *et al.*, 1998). Estas respostas sugerem uma hipersensibilização da fibra nervosa causada por agentes que modulam a ativação ou retardo do mecanismo de portão dos canais de sódio voltagem-dependentes (ARMSTRONG, 1992; CATTERALL, 1992). Como suporte a estes dados, a seqüência de aminoácidos obtida da fração ativa da peçonha da *P. chrysothorax* assemelha-se em estrutura molecular e carga isoelétrica, além dos efeitos fisiológicos, com as α- e β-PMTX, isoladas das peçonhas das vespas solitárias *Anoplius*

samariensis e *Batozonellus maculifrons*, respectivamente, e que têm ação pré-sináptica, desativando a corrente de sódio de axônios de artrópodes retardando o mecanismo de portão dos canais de sódio (KONNO *et al.*, 1997, 1998, 2000; SAHARA *et al.*, 2000).

Vários estudos já demonstraram que peçonhas de vespas contêm neurotoxinas que são ativas principalmente em preparações neuromusculares de insetos (PIEK 1982; KITS e PIEK, 1986; KONNO *et al.*, 1997, 1998). Além disso, alguns estudos também revelam a ação neurotóxica destas peçonhas em preparações de mamíferos (EDERY, 1972; HARSCH *et al.*, 1998; KONNO *et al.*, 1998; OLIVEIRA e PALMA, 1998; SAHARA *et al.*, 2000; YOKOTA *et al.*, 2001; MIYAWAKI *et al.*, 2002). Por exemplo, a hornetina, da peçonha da *Vespa flavitarssus*, tem ação pré-sinaptica em preparações biventer cervicis de pintinho e em nervo frênico-diafragma de camundongo (HO e KO, 1986). Mais recentemente, NABIL *et al.* (2002) relataram que peçonhas das vespas solitárias *Bembix oculata, Dielis collaris* e *Scolia erythrocephala* exercem ações tóxicas em diferentes tipos de músculos de mamíferos.

Diante disso, investigamos se as peçonhas apresentavam efeitos significativos em preparações de mamíferos (artigo 4; pág. 127), sendo utilizadas para isso preparações de junção neuromuscular de camundongos. No nervo frênico-diafragma de camundongos, as peçonhas de *P. chrysothorax* e *P. compressa* apresentaram maiores efeitos sobre a neurotransmissão quando comparadas às outras peçonhas estudadas neste trabalho (*P. rejecta*, *Pypper* sp1 e *Pypper* sp2, ver Resultados Adicionais), causando bloqueios de 80% à 90% das respostas contráteis. As peçonhas não apresentaram ações nas preparações de biventer cervicis de aves (resultados não mostrados). Nas análises eletrofisiológicas realizadas com a peçonha de *P. chrysothorax*, houve uma significativa despolarização induzida tanto na região da placa terminal quanto em regiões distantes da placa. Estas observações, somadas ao bloqueio da resposta contrátil indireta em preparações

nervo frênico-diafragma de camundongo e ao bloqueio da resposta da ACh exógena em preparações de diafragma cronicamente desnervado, sugerem a princípio um efeito pós-sinaptico da peçonha nos receptores nicotínicos, considerando ainda os danos nas fibras musculares, possivelmente causados pela ação miotóxica das fosfolipases (GUTIÉRREZ e LOMONTE, 1995; OWNBY *et al.*, 1997; OLIVEIRA e PALMA, 1998).

As ações despolarizantes de varias toxinas nas regiões juncionais e não juncionais da membrana muscular, bem como a distribuição dos canais de sódio envolvidos nestes eventos que extendem-se tanto nas regiões sinápticas quanto ao longo da fibra muscular, com maior densidade próximo à placa motora, foram discutidas por VITAL BRAZIL e FONTANA (1993). Uma ação pré-sináptica pode levar à despolarização da membrana do axônio (FONTANA e VITAL-BRAZIL, 1985; GRÉGIO *et al.*, 1999), enquanto que uma ação pós-sináptica pode afetar os canais de sódio da membrana da fibra muscular, os receptores nicotínicos da região da placa, e causar dos danos no músculo (EDERY *et al.*, 1972; VITAL BRAZIL e FONTANA, 1993). Estes mecanismos de ação sugerem a presença na peçonha de *P. chrysothorax* de uma toxina ativa sobre canais de sódio da junção neuromuscular.

CONCLUSÕES

CONCLUSÕES

1- Das peçonhas investigadas neste estudo, apenas as espécies sociais *Polybia chrysothorax*, *Pseudopolybia compressa* e *Polybia rejecta* apresentaram ações expressivas e significantes nas preparações de artrópodes e mamíferos empregados.

2- Os resultados obtidos neste estudo sugerem que as toxinas presentes nas peçonhas das vespas *P. chrysothorax* e *P. compressa* atuam sobre os canais de Na^+ voltagem dependentes da fibra nervosa e muscular de insetos.

3- A seqüência obtida (ARSLLEGLGIRRGSA) para uma toxina da peçonha da *P. chrysothorax* indicou que o peptídeo pertence à família de peptídeos conhecidos como pompili dotoxinas (PMTX), a qual contém duas isoformas, α-PMTX e β-PMTX, purificados das peçonhas de vespas solitárias.

4- A demonstração de que as ações cardio- e neurotóxicas da peçonha de *P. chrysothorax* eram devidas em grande parte à presença de uma isoforma de PMTX sugere que peçonhas de espécies de vespas sociais podem servir de fontes para moléculas com potencial utilidade como inseticidas.

SUMMARY

SUMMARY

Wasp venoms produce a variety of biological effects, including pain, edema, hypotension, hepatotoxicity and allergic reactions. With the exception of bee (*Apis mellifera*) venom and of a few wasp species, little is known of the toxinology of Brazilian hymenopterans. In this work, we examined the pharmacological activities of venom from three species of social wasps (*Polybia chrysothorax*, *Pseudopolybia compressa* and *Polybia rejecta*, family Vespidae) and two species of solitary wasps of the genus *Pypper* (family Scoliidae) found in northeastern Brazil. Specifically, we evaluated the activity on smooth muscle (guinea pig isolated ileum), the cardiotoxicity in cockroach semi-isolated heart, and the action on neurotransmission in crustacean (*Callinectes danae*, blue crab) sensorial nerve and in mouse phrenic nerve-diaphragm preparations.

The venoms of the three social species (0.001-100 μg/ml, depending on the venom) contracted guinea pig isolated ileum, with EC_{50} of 0.25 ± 0.05 μg/ml, 8.75 ± 1.25 μg/ml and 7.65 ± 1.85 μg/ml (mean±SEM, n=4) for *P. chrysothorax*, *P. rejecta* and *P. compressa*, respectively. The venoms of the two species of *Pypper* were inactive in this tissue. Pre-incubating the tissues with atropine (antagonist of muscarinic receptors, 10 μM), methysergide (antagonist of serotonin 5-HT_2 receptors, 10 μM), and pyrilamine (antagonist of histamine H_1 receptors, 10 μM) inhibited the contractile activity of the three social venoms, whereas Hoe-140 (antagonist of bradykinin B_2 receptors, 1 μM) had no effect on the responses. These results indicate that the social wasp venoms contained amines (acetylcholine, histamine and serotonin) but not kinins.

The three venoms (1-100 μg) showed cardiotoxicity in cockroach semi-isolated heart, with the most potent being *P. chrysothorax* and *P. compressa*; the venoms of the

genus *Pypper* were inactive in this preparation. Investigation of the mechanisms responsible for this effect based on the use of antagonists/inhibitors indicated that there was no contribution by venom amines and that the most probable cause was an action involving ion channels, especially sodium channels and, to a lesser degree, potassium channels. The venoms showed the following order of potency for their phospholipase A activity: *P. chrysothorax/P. compresas*> *P. rejecta* >> *Pypper* spp. However, this activity contributed little to the observed effects since indomethacin only delayed the cardiotoxicity but did not abolish it.

The venoms of *P. chrysothorax* and *P. compressa* (100 μg each) increased the spontaneous electrical activity of crustacean sensorial nerve in a manner similar to that observed with brevetoxin ($5x10^{-7}$ M), a marine toxin that affects the gating mechanism of voltage-dependent sodium channels. These venoms also inhibited sensory nerve action potentials in sucrose gap experiments. This effect was abolished by tetrodotoxin (10^{-6} M), a blocker of voltage-dependent sodium channels. The venoms of *P. rejecta* and of the genus *Pypper* were inactive in this preparation.

The venom of the three social species (25-100 μg/ml), but not the solitary species, inhibited neurotransmission in mouse phrenic nerve-diaphragm preparations (the time for 50% blockade of the contractile responses was 40 ± 5 min and 57 ± 3 min for *P. chrysothorax* and *P. compressa*, respectively, at 100 μg/ml; n=3 each). This inhibition was irreversible by washing the preparations. The social wasp venoms also depolarized the muscle fiber membrane and the terminal endplate of neuromuscular junctions. These observations suggested a possible postsynaptic action for the venoms, with some presynaptic involvement. The three social venoms also produced morphological alterations

in muscle that were compatible with myonecrosis. The level of this damage correlated with the venom PLA_2 activity.

The fractionation of *P. chrysothorax* and *P. compressa* venoms by RP-HPLC on a C_{18} column resulted in several peaks. The main peak of *P. chrysothorax* venom was sequenced. The sequence obtained (ARSLLEGLGIRRGSA) indicated that the peptide belonged to the family of peptides known as pompilidotoxinas (PMTX), which contains two isoforms, α-PMTX and β-PMTX, purified of the venoms of the soltary wasps *Anoplius samariensis* and *Batozonellus maculifrons*, respectively. This toxin reproduced the cardiotoxicity in cockroach heart and the blockade of neurotransmisson in crustacean nerves seen with *P. chrysothorax* venom. The results of this study indicate that the venoms investigated had several biological activities. The demonstration that the cardio– and neurotoxicity of *P. chrysothorax* venom were largely attributable to the presence of a novel isoform of PMTXs suggests that venom social wasp of species may serve as a source of molecules with potential usefulness as insecticides.

REFERÊNCIAS BIBLIOGRÁFICAS

REFERÊNCIAS BIBLIOGRÁFICAS

ABE, T.; HARIYA, Y.; KAWAI, N.; NIWA, A. (1989) Comparative study of amino acid composition in an extract from hornet venom sacs: high content of neuroactive amino acids in *Vespa*. **Toxicon** 27, 683-688.

ABE, T.; KAWAI, A. (1983) Cardioactive effects of hornet venom (*Vespa mandarina*). **Comp. Biochem. Physiol**. 76, 221-228.

ABE, T.; KAWAI, N.; NIWA, A. (1982) Purification and properties of a presynaptically acting neurotoxin, mandaratoxin, from hornet (*Vespa mandarina*). **Biochemistr**. 21, 1693-1697.

ADAMS, M. E.; BINDOKAS, V. P.; HASEGAWA, L.; VENMA, V. J. (1990) ω-Agatoxin: novel calcium channel antagonists of two subtypes from funnel spider (*Agelenopsis aperta*) venom. **J. Biol. Chem**. 265, 861-867.

ALLALOUF, D.; BER, A.; ISHAY, J. (1972) Hyaluronidase activity of extracts of venom sacs of a number of Vespinae (hymenoptera). **Comp. Biochem. Physiol**. 43 B, 119-123.

AMAT, C.; SINAKEVITCH-PEAN, I.; HUE, B. (1997) Proctolin-like immunoreactivty in dorsal paired media neurons generating plateau potenceals in cockroach *Periplaneta americana*. **Neurosc. Res.** 29, 263-268.

ARMSTRONG, C. M. (1981) Sodium channel and gating currents. **Physiol. Rev.** 61, 644-683.

ARMSTRONG, C. M. (1992) Voltage-dependent ion channels and their gating. **Physiol. Rev.** 72, S5-S13.

AZEVEDO-MARQUES, M. M.; FERREIRA, D. B.; COSTA, R. S. (1992) Rhabdomyonecrosis experimentally induced in Wistar rats by Africanized bee venom. **Toxicon** 30, 344-348.

BALAJI, R. A.; SASAKI, T.; GOPALAKRISHNAKONE, P.; SATO, K.; KINI, R. M.; BAY, B. H. (2000) Purification, structure determination and synthesis of covalitoxin-II, a short insect-specific neurotoxic peptide from the venom of *Coremiocnemis validus* (Singapore tarantula). **FEBS Lett.** 474, 208-212.

BARNES, R. D.; RUPPERT, E. E. (1994) **Invertebrate Zoology**. 6th ed. Saunders College Publishing: Orlando. pp. 837-838.

BAUMANN, E.; GERSCH, M. (1982) Purification and identification of neurohormone D, a heart accelerating peptide from the corpora cardiaca of the cockroach *Periplaneta americana*. **Insect Biochem**. 12, 7-14.

BEGHINI, D. G.; TOYAMA, M. H.; HYSLOP, S.; SODEK, L.; NOVELLO, J. C.; MARAGONI, S. (2000) Enzymatic characterization of a novel phospholipase A_2 from *Crotalus durissus cascavella* rattlesnake (Maracambóia) venom. **J. Protein Chem**. 19, 603-607.

BEN KALIFA, C.; STANKEIWICZ, M.; PELHATES, M.; SERRANO-HERNANDEZ, S. E.; POSSANI, L. D.; HINKEL, H.; MEBS, D. (1997) Action of babycurus-toxin 1 from the East african scorpion *Babycurus centrurimorphus* on the isolated cockroach giant axon.**Toxicon**. 35, 341-361

BLAGBROUGH, I. S.; BRACKLEY, P. T. H.; BRUCE, M.; BYCROFT, B. W.; MATHER, A. J.; MILLINGTON, S.; SUDAN, H. L.; USHERWOOD, P. N. R. (1992) Arthropod toxins as leads for novel insecticides: an assessment of polyamine amides as glutamate antagonists. **Toxicon**. 30, 303-322.

BLASCHKE, M.; KELLER, B. U.; RIVOSECCHI, R.; HOLLMANN, M.; HEINEMANN, S.; KONNERTH, A. (1993) A single amino acid determines the subunit-specific spider toxin block of -amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor channels. **Proc. Natl. Acad. Sci. U.S.A.** 90, 6528-6532.

BROWN, B. E.; STARRAT, A. N. (1975) Isolation of proctolin, a myotropic peptide, from *Periplaneta americana*. **J. Insect Physiol**. 21, 1879-1881

BRUCE, M.; ELDEFRAWI, A. T.; ELDEFRAWI, M. E.; GOODNOW, R.; KONNO, K.; NAKANISHI, K.; USHERWOOD, P. N. R. (1990) Struture-activity relationships of analogues of the wasp toxin philanthotoxin: non-competitive antagonists of quisqualate receptors. **Toxicon** 28, 1333-1346.

BÜLBRING, E. (1946) Observations on the isolated phrenic nerve-diaphragm preparation of the rat. **Br. J. Pharmacol**. 1, 38-61.

BUZIN, M. P.; HYSLOP, S. (1999) Atividades enzimáticas e biológicas do extrato de sacos de veneno da vespa social *Polistes lanio lanio*. Dissertação de Mestrado, Faculdade de Ciências Médicas. UNICAMP-SP.

CASSOLA, A. C.; AFECHE, S. C. (1996) Use of neurotoxins to study Ca^{2+} channel functions. **Braz. J. Med. Biol. Res**. 29, 1759-1763.

CATTERALL, W. A. (1992) Cellular and molecular biology of voltage-gated sodium channels. **Physiol. Rev**. 72, S15-S48.

CATTERALL, W. A. (1993) Structure and function of voltage-gated ions channels. **Trends Neurosci**. 16, 500-506.

CHUANG, C. C.; HUANG, W. C.; YU, H. M.; WANG, K. T.; WU, S. H. (1996) Conformation of *Vespa basalis* mastoporan-B in trifluoroethanol-containing aqueous solution. **Biochim. Biophys. Acta** 1292, 1-8.

COSTA, H.; PALMA, M. S. (2000) Agelotoxin: a phospholipase A_2 from the venom of the neotropical social wasp cassununga (*Agelaia pallipes pallipes*) (Hymenoptera – Vespidae). **Toxicon** 38, 1367-1379.

CURTIS, C. R.; WALKINS, J. C. (1965) The pharmaclogy of amino acids related to gamma-amino butyric acid. **Pharmac. Rev.** 17, 347- 352

CUTHBERT, B. A.; EVANS, P. D. (1989) A comparison of the effects of FRMFamide-like peptide on locust heart and skeletal muscle. **J. Exp. Biol.** 144, 395-415.

DEAL, K. K.; ENGLAND, S. K.; TAMKUN, M. M. (1996) Molecular physiology of cardiac potassium channels. **Physiol. Rev.** 76, 49-67.

DREYER, F. (1990) Peptide toxins and potassium channels. **Rev. Physiol. Biochem. Pharmacol.** 115, 94-140.

DUVE, H.; WREN, P.; THORPE, A. (1995) Innervation of the foregut of the cockroach Lueuphaea maderse and inhibition of spontaneous contractile activity by collatostain neuropeptides. **Physiol. Entomol.** 20, 33-44.

DUVE, H.; ELIA, A. J.; ORCHARD, I.; JOHSEN, A. H.; THORPE, A. (1993) The effects of calliFMRFarmides and other FMRFamide-related neuropeptides on the activity of the heart of the bloufly *Calliphora vomitoria*. **J. Insect. Physiol.** 39, 31-40.

EAST, P. D.;, HALES, D. F.; COOPER, P. D. (1997) Distribution of sulfakinin-like peptide in the central and sympathetic nervous system of the American cockroach, *Periplaneta americana* (L.) and field cricket, *Teleogrullus commodus* (Walker). **Tissue Cell.** 29, 347-354.

ECKERT, M.; PREDEL, R.; GUNDEL, M. (1999) Periviscerokinin-like immunoreactivity in the nervous system of the American cockroach. **Cell Tissue Res.** 295, 159-170.

EDERY, H.; ISHAY, J.; GITTER, S. (1972) Pharmacological activity of oriental hornet (*Vespa orientalis*) venom. **Toxicon** 10, 13-23.

EDERY, H.; ISHAY, J.; GITTER, S.; JOSHUA, H. (1978) Venoms of Vespidae. In: *Arthopod Venoms.* **Handbook of Experimental Pharmacology** Vol. 52. pp. 691-771. Spinger Verlag: Berlin.

ELDEFRAWI, A. T.; ELDEFRAWI, M. E.; KONNO, K.; MANSOUR, N. A.; NAKANISHI, K.; OLTZ, E.; USHERWOOD, P. N. R. (1988) Structure and synthesis of a potent glutamate receptor antagonist in wasp venom. **Proc. Natl. Acad. Sci. U.S.A.** 85, 4910-4913.

ENTWISTLE, I. D.; JOHNSTONE, R. A. W.; MEDZIHRADAZKY, D.; MAY, T. E.. (1982) Isolation of a pure toxic polypeptide from the venom of the spider *Phoneutria nigriventer* and its neurophysiological activity on an insect fermur preparation. **J. Exp. Biol.** 20, 3531-3545.

FAN, H. W.; FRANÇA, F. S. O (1992) Acidentes por himenópteros. Em: **Plantas Venenosas e Animais Peçonhentos** (Schvartsman S, ed.). pp. 228-232. Editora Sarvier: São Paulo.

FIGUEIREDO, S. G.; LIMA, M. E.; CORDEIRO, M. N.; DINIZ, C. R.; PATTEN, D.; HALLIWELL, R. F.; GILROY, J.; RICHARDSON, M. (2001) Purification and amino acid sequence of a highly insetcidal toxin from the venom of the Brazilian spider *Phoneutria nigriventer* which inhibits NMDA-evoked currents in rat hippocampal neurones. **Toxicon.** 39, 309-317.

FONTANA, M. D.; VITAL-BRAZIL, O. (1985) Mode of action of *Phoneutria nigriventer* spider venom at the isolated phrenic nerve-diaphragm of the rat. **Braz. J. Med. Biol. Res.** 18, 557-565.

FOZZARD, H. A.; HANCK, D. A. (1996) Structure and function of voltage-dependent sodium channel: comparison of brain II and cardiac isoforms. **Physiol. Rev.** 76, 887-925.

FREITAS, J. C.; OGATA, M.; KODAMA, M.; MARTINEZ, S. C. G.; LIMA, M. F.; MONTEIRO, C. K. (1992) Possible microbial source of guanidine neurotoxins found in the mussel *Perna perna* (molusca, bivalvia, Mytilidae). Gopala krishnakonep, Tan C.K. (Ed.). **Recent Advances Toxinol**. *Res.* 2, 589-596.

FRONTALI, N.; CECCARELLI, B.; GORIO, A.; MAURO, A.; SIEKEVITZ, P.; TZENG, M. C. (1976) Purification from black widow spider venom of protein factor causing the depletion of synaptic vesicles at neuromusclar junctions. **J. Cell Biol**. 68, 462-479.

GAWADE, S. P. (1983) The effect of venom from the Indian tropical wasp *Vespa tropica* on nerve-muscle preparations from *Drosophila* larvae. **Toxicon** 21, 882-886.

GUTIÉRREZ, J. M.; LOMONTE, B. (1995) Phospholipase A2 myotoxins from Bothrops snake venoms.**Toxicon** 33, 1405-1224.

GOODMAN, A.; GOODMAN, L.; GILMAN, A. (1996) **The Pharmacological Basis of Therapeutics**. 9nd ed. Macmilllan Publishing.

GRIESBACKER, T.; ALTHUBER, P.; ZENZ, M.; RAINER, I.; GRIENGL, S.; LEMBECK, F. (1998) *Vespula vulgaris* venom: role of kinins and release of 5-hydroxytryptamine from skin mast cells. **Eur. J. Pharmacol**. 351, 95-104.

GUYTON, A. C.; HALL, J. E. (2000) **Textbook of Medical Physiology**.10nd ed. Copyriht.

HABERMANN E (1972) Bee and wasp venoms. **Science** 177, 314-322.

HAMES, B. D. (1990) One-dimensional polyacrylamide gel electrophoresis. In: *Gel* **Electrophoresis of Proteins- a Practical Approach** (Hames BD, Rickwood D, eds). 2nd ed. pp. 1-147. IRL Press: Oxford.

HENRIKSE, A.; KING, T. P.; MIRSA, O.; MONSALVE, R. I.; MENO, K.; IPSEN, H.; LARSEN, J. N.; GAJHEDE, M.; SPANGFORT, M. D. (2001) . Major venom allergen of yellow jacket, Ves v 5: structural chracterization of a pathogenesis protein superfamily. **Proteins** 45, 438-448.

HIGASHIJIMA, T.; UZU, S.; NAKAJIMA, T.; ROSS, E. M. (1998) Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). **J. Biol. Chem.** 263, 6491-6494.

HILL, E. S.; BLAGBURN, J. M. (2001) Presynaptic effects of biogenic amines modulating synaptic transmission between identified sensory neurons and giant interneurons in the first instar cockroach. **J. Comp. Physiol.** 187, 633-645.

HO, C. L.; CHEN, W. C.; LIN, Y. L. (1998) Structures and biological activities of new wasp venom peptides isolated from the black-bellied hornet *(Vespa basalis)* venom. **Toxicon** 36, 609-617.

HO, C. L.; HWANG, L. L.; LIN, Y. L.; CHEN, C. T.; YU, H. M.; WANG, K. T. (1994) Cardiovascular effects of mastoparan B and its structural requirements. **Eur. J. Pharmacol.** 259, 259-264.

HO, C. L.; KO, J. L. (1988) Purification and characterization of a lethal protein with phospholipase A_1 activity from the hornet (*Vespa basalis)* venom. **Biochim. Biophys. Acta** 963, 414-422.

HOFFMAN, D. R. (1985) Allergens in Hymenoptera venom XIII: isolation and purification of protein components from three species of vespid venoms. **J. Allergy Clin. Immunol.** 75, 599-605.

HORI, S.; KAWAI, N.; NIWA, A.; OHOTANI, S. (1977) Separation of neurotoxins from hornet *(Vespa insularis)* venom and their actions on crustacean neuromuscular transmission. **J. Neurochem.** 28, 1183-1188.

JEGLITSCH, G.; REIN, K.; BADES, D. G.; ADAMS, D. J. (1990) Brevetoxin-3 (PbTx-3) and its derivates modulate single tetrodotoxin-sensitive sodium channels in rat sensory neurons. **Pharmacol.** 284, 516-525.

KAWAI, N.; ABE, T.; HORI, S.; NIWA, A. (1980) Effects of neurotoxin in hornet venom on neuromuscular transmission of lobster. **Comp. Biochem. Physiol.** 65C, 87-92.

KAWAI, N.; MAURO, A.; GRUNDFEST, H. (1972) Effects of black widow spider venom on the lobster neuromuscular juntions. **J. Gen. Physiol**. 60, 650-664.

KING, T. P.; KOCHOUMIAN, L.; JOLYN, A. (1984) Wasp venom proteins: phospholipase A_1 and B. **Arch. Biochem. Biophys**. 230, 1-12.

KING, T. P.; LU, G.; GONZALEZ, M.; QIAN, N.; SOLDATOVA, L. (1996) Yellow jacket venom allergens, hyaluronidase and phospholipase: Sequence similarity and antigenic cross-reactivity with their hornet and wasp homologs and possible implications for clinical allergy. **J. Allergy Clin. Immunol**. 98, 588-600.

KITS, K. S.; PIEK, T. (1986) Action of the polyamine β-philanthotoxin on neuromuscular transmission in insects. **Neuropharmacology** 25, 1089-1093.

KLOWDEN, M. J.; VITALE, A. J.; TRUMBLE, M. J.; WESSON, C. R.; TRUMBLE, W. R. (1992) A bioassay for cobra cardiotoxin activity using semi-isolated cockroach heart. **Toxicon** 30, 295-301.

KONNO, K.; HISADA, M.; ITAGAKI, Y.; NAOKI, H.; NOBUFUMI, K.; MIWA, A.; YASUHARA, T.; TAKAYAMA, H. (1998) Isolation and structure of pompilidotoxins, novel peptide neurotoxins in solitary wasp venoms. **Biochem. Biophys. Res. Commun**. 250, 612-616.

KONNO, K.; HISADA, M.; NAOKI, H.; ITAGAKI, Y.; YASHARA, T.; JULIANO, M. A.; JULIANO, L.; PALMA, M. S.; YAMANE, T.; NAKAJIMA, T. (2001) Isolation and sequence determination of peptides in the venom of the spider wasp *Cyphononyx dorsalis* guided by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. **Toxicon**. 39, 1257-1260.

KONNO, K.; HISADA, M.; NAOKI, H.; ITAGAKI, Y.; YASUHARA, T.; NAKATA, Y.; MIWA, A.; KAWAI, N. (2000) Molecular determinants of binding of a wasp toxin (PMTXs) and its analogs in the Na^{+} channels proteins. **Neurosc. Lett.**. 285, 29-32.

KONNO, K.; MIWA, A.; TAKAYAMA, H.; HISADA, M.; ITAGAKI, Y.; NAOKI, H.; YASUHARA, T.; KAWAI, N. (1997) α- Pompilidotoxin (α-PMTX), a novel neurotoxin from the venom of a solitary wasp, facilitates transmission in the crustacean neuromuscular synapse. **Neurosci. Lett**. 238, 99-102.

KONNO, K.; PALMA, M.S.; HITARA, I. Y.; JULIANO, M. A.; JULIANO, L.; YASUHARA, T. (2002) Identification of bradykinins in solitary wasp venoms. **Toxicon**. 40, 309-312.

LAGOS, P.; DURAN, R.; CERVENANSKY, C.; FREITAS, J. C.; SILVEIRA, R. (2001) Identification of hemolytic and neuroactive fractions in the venom of the sea anemone *Budosoma cangicum*. **Braz. J. Med. Biol. Res**. 34, 895-902.

LANGE, A. B.; BENDENA, W. G.; TOBE, S. S. (1995) The effect of the thirteen Dip-allatostatins on myogenic and induced contractions of the cockroach (*Diploptera puncata*) hindgut. **J. Insect Physiol.** 41, 581-588.

LI, Y. J.; TAN, Z. Y.; JI, Y. H. (2000) The binding of BmK IT2, a depressant insect-selective scorpion toxin on mammal and insect sodium channels. **Neurosc. Res.** 38, 257-264.

LITTER, S.; WYPYCH, J. I.; NOBLE, R. W.; ABEYOUNIS, J. C.; REISMAN, R. E. (1985) Allergenic components of bald-faced hornet (*V. maculata*) venom. **Int. Archs. Allergy Appl. Immunol.** 76, 1-8.

LONGENECKER, H. E. J.; HURLBUT, W. P.; MAURO, A.; CLARK, A. W. (1970) Effect of black widow spider venom on the frog neuromuscular junction. **Nature**, 225, 701-703.

MAC PHERSON, M. R.; POLLOCK, V. P.; BRODERICK, K. E.; KEAN, L.; O'CONNELL, F. C.; DOW, J. A. T.; CAVIES, S. A. (2001) Model organisms: new insights into ion channel and transporter function. L-type calcium channels regulate epithelial fluid transport in *Drosophila malenogaster*. **Am. J. Physiol. Cell. Physiol.** 280, 394-415.

MALPEZZI, E. L. A.; FREITAS, J. C.; MURAMOTO, K.; KAMIYA, H. (1993) Characterzation of peptides in sea anemone venom collected by novel procedure. **Toxicon**. 31, 853-864.

MILLER, T. (1979) Nervous versus neurohormonal control of insect heartbeat. **Am. Zool**. 19, 77-86.

MINTZ, I. M.; VENMA, V. J.; SWINDEREK, K. M.; LEE, T. D.; BEAN, B. P.; ADAMS, M. E. (992) P-type calcium channels blocked by the spider toxin ω-Aga-IV. **Nature**, 355, 827-829.

MUELLER, A. L.; ROELOFFS, R.; JACKSON, H. (1995) Pharmacology of polyamine toxins from spiders and wasps. In **The Alkaloids. Chemistry and Pharmacology**; Cordell, G. A., Ed.; Academic Press: San Diego,; Vol. 46; pp 63-94.

NABI, Z. I., ZALAT, S., ABO-GHALIA, A. H., ELBANNA, S. (2002) Pharmacology and chemistry of the venoms of solitary wasp. **J. Nat. Toxins**. 11, 15-24.

NAKAJIMA, T. (1984) Biochemistry of vespid venom. In: **Insect and Arthropod Venoms**. Tu AT (ed.). pp. 110-133. Marcel Dekker: New York.

NAKAJIMA, T.; YASUHARA, T.; UZU, S.; WAKAMATSU, T.; FUKUDA, K.; TSUKAMOTO, Y. (1985) Wasp kinins, new cytotrophic peptide families and their physico-chemical properties. **Peptides** 6, 425-430.

NEUMCKE, B. (1990) Diversity of sodium channels in adult and cultured cells, in oocytes and lipid bilayers. **Rew. Physiol. Biochem. Pharmacol.** 151, 1-49.

O'CONNOR, R.; PECK, M. L. (1978) Venoms of Apidae. In: **Arthopod Venoms. Handbook of Experimental Pharmacology** (Bettini, S, ed.). pp. 613-659. Spinger Verlag: Berlin.

OLIVEIRA, M. R.; PALMA, M. S. (1998) Polypia: a group of phospholipases A_2 from the venom of the neotropical social wasp paulistinha (*Polybia paulista*). **Toxicon**. 36, 189-199.

PASS, G.; SPERK, G.; AGRICOLA, H.; BAUMANN, E.; PENZLIN, H. (1988) Octopamine in a neurohaemal area within the antenral heart of the American cockroach. **J. Exp. Biol.** 135, 495-498.

OWNBY, C. L., POWELL, J. R., JIANG, M. FLETCHER, J.E. (1997) Melittin and phospholipase A_2 from bee (*Apis mellifera*) venom cause necrosis of murine skeletal muscle in vivo. **Toxicon**. 35, 67-80.

PELHATE, M.; ZLOTKIN, E. (1982) Actions of insect toxin and other toxins derived from the venom of the scorpion *Androctonus australis* on isolated giant axons of the cockroach (*Periplaneta americana*). **J. Exp. Biol.** 97, 67-77.

PELZER, D.; PELZER, S.; MACDONALD, T. F. (1990) Properties and regulation of calcium channel in muscle cells. **Rev. Physiol. Biochem.** 114, 108-207.

PIEK, T. (1982) δ-Philanthotoxin, a semi-irreversible blocker of ion channels. **Comp. Biochem. Physiol.** 72C, 311-315.

PIEK, T. (1984) Pharmacology of Hymenoptera venoms. In: **Venoms of Arthropods Handbook of Natural Toxins** Vol. 2 (Tu AT, ed.). pp. 135-185. Marcel Dekker: New York.

PIEK, T. (1991) Neurotoxic kinins from wasp and ant venoms. **Toxicon** 29, 139-149.

PIEK, T.; SIMON, S.; THOMAS, R. T. (1969) Paralysing venoms of solitary wasps. **Comp. Biochem. Physiol.** 30, 13-31.

PIRPIGNANE, M. L.; RIVER, E.; HELLMAN, U.; BISCOGLIO DE JIMENEZ BONINO, M. (2002) Structural and immunological aspects of *Polybia scutellaris* Antigen 5. **Arch Biochem Biophys** 407, 224-230.

POSSANI, L. D. (1984) Structure of scorpion toxins. In: *Handbook of Natural Toxins. Vol. 2. Insect Poisons, Allergens and other Invertebrate Venoms* (Tu AT, ed). pp. 513-550. Marcel Dekker: New York.

PREDEL, R.; AGRICOLA, H.; LINDE, D.; WOLLWEBER, L.; VEESTRA, J. A.; PENZLIN, H. (1994) The insect neuropeptide corazonin: physiological and immunocytochemical studies in **Blattyarie. Zoology** 98, 35-49.

PREDEL, R.; KELLNER, R.; RAPUS, J.; NACHMAN, R.; GÄDE, G. (1999) Post-translational modifications of the insect sulfakinins. **Eur. J. Biochem**. 263, 552-560.

QUISTAD, G. B.; REUTER, C. C.; SKINNER, W. S.; DENNIS, P. A.; SUWANRUMPHA, S.; FU, E. W. (1991) Paralytic and insecticidal toxins from the funnel web spider, *Hololena curta.* **Toxicon** 29, 329-336.

RANG, H. P.; DALE, M. M.; RITTER, J. M. (1995) **Pharmacology**. 3rd ed. Blackwell Publishers: London.

RICHARDS, I. S.; KULKARNI, A. P.; BROOKS, S. M.; PIERCE, R. (1990) Florida red-tide toxins (brevetoxins) produce depolarization of airway smooth muscle. **Toxicon** 28, 1105-1111.

ROGERS, J. C.; QU, Y.; TANADA, T. N.; SCHEUER, T.; CATTERALL, W. A. (1996) Molecular determinants of high affinity binding of α-scorpion toxin and sea anemone toxin in the S3-S4 extracellular loop in domain IV of the sodium channel α subunit. **J. Biol. Chem.** 271, 15950-15962.

SAHARA, Y.; GOTOH, M.; KONNO, K.; MIWA, A.; TSUBOKAWA, H.; ROBINSON-HUGH, P. C.; KAWAI, N. (2000) A new class of neurotoxin from the slows inactivation of sodium current. **Eur. J. Neurosc.** 12, 1961-1970.

SAUTIÈRE, P.; CESTÈLE, S.; KOPEYAN, C.; MARTINAGE, A.; DROBECQ, H.; DOLJANSKY, Y.; GORDON, D. (1998) New toxins acting on sodium channels from the scorpion Leiurus quinquestriatus *Hebraeus* suggest a clue to mammalian vs insect selectivity. **Toxicon.** 36, 1141-1154.

SCHMIDT, J. O. (1994) Toxinology of venoms from the honeybee genus *Apis*. **Toxicon** 33, 917-927.

SCHMIDT, J. O.; BLUM, M. S.; OVERAL, W. L. (1986) Comparative enzymology of venoms from stinging Hymenoptera. **Toxicon.** 24, 907-921.

SCHREIBMAYER, W.; JEGLITSCH, G. (1992) The sodium channel activator brevetoxin-3 uncovers a multiplicity of different open states of cardiac sodium channels. **Biochim. Biophys. Acta** 1104, 233-242.

SEVCIK, C.; ALVAREZ-VASQUEZ, F.; SAAVEDRA, J. Á.; CORDOVEZ, G. (1986) Blockage of resting potassium conductance in frog muscle fibers by a toxin isolated from the sponge *Haliclona viridis*. **Toxicon**. 24, 851-860.

SLIWOWSKA, J.; ROSINSKI, G.; NASSEL, D. R. (2001) Cardioacceleratory action of tachykinin-related neuropeptides and proctolin in two coleoptera insect species. **Peptides** 22, 209-217.

STANKIEWICZ, M.; PANEK, I.; GRAJPEL, B.; TEGOWSK. E.; KADZIELA, W.; PELHATE, M. (2000) Modification of bioelectric functions in the nervous system as a result of neurotoxin action. **Pastepy Hig. Med. Dosw**. 54, 371-379.

STRMGAARD, K.; ANDERSEN, K.; KROGSGAARD-LARSEN, P.; JAROSZEWSKI, J. W. (2001) Recent advances in the medicinal chemistry of polyamine toxins. **Mini-Rev. Med. Chem.** 1, 317-338.

TAKASAKI, C.; FUKUMOTO, M. (1989) Phospholipase B from venom japae yellow hornet (*Vespa xanthotera*) venom. **Toxicon**. 24, 449-485.

THE, STAFF. (1968) **Pharmacological Experiments on Isolated Preparations**. Livingstone E&S: Edinburgh.

TRAINA, M. E.; BELLINO, M.; SERPIETRI, L.; MASSA, A.; FRONTALI, N. (1976) Heart-accelerating peptides from cockroach corpor cardiaca. **J. Insect Physiol**. 22, 323-329.

TSIEN, R. W.; BEAN, B. P.; HESS, P.; LANSMAN, J. B.; NILIUS, B.; NOWYCKY, M. C. (1986) Mechanisms of calcium channel modulation by β-adrenergic agents and dihydropyridine calcium agonists. **J. Mol. Cell Cardiol**. 18, 691-710.

USHERWOOD, P. N. R. (1967) Insect neuromuscular mechanisms. **Am. Zool.**. 7, 553-582.

USHERWOOD, P. N. R.; MACHILI, P. (1968) Pharmacological properties of excitatory neumuscular synapses in the locust. **J. Exp. Biol**. 49, 341-361

USHERWOOD, P. N. R.; MICHILI, P.; LAEF, G. (1977) L-Glutamate at insect excitatory nerve-muscle synapses. **Nature** 219, 1169-1180.

VARGAS-VILLARREAL, J.; MARTIN-POLO, J.; REYNAUD, E.; ALAGON, A. C. (1991) A new affinity adsorbent for the purification of phospholipases A_1 and A_2 from animal venoms. **Toxicon**. 29, 119-124.

VILAPLANA, L.; MAESTRO, J. L.; PIULACHS, M. D.; BELLÉS, X. (1999) Modulation of cardiac rhythm by allostatins in the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). **J. Insect Physiol**. 45, 1057-1064.

VISSER, B. J.; LABRUYÈRE, W. T.; SPANJER, W.; PIEK, T. (1983) Characterization of two paralysing protein toxins (A-MTX and B-MTX), isolated from a homogenate of the wasp *Microbracon hebetor* (Say). **Comp. Biochem. Physiol.** 75B, 523-530.

WATSON, J.; RITZMANN, R. E. (1995) Combined intracellular stimulation and high video motion analysis of motor control neurons in the cockroach. **J. Neurosci Meth.**. 61, 151-157.

WEGENER, C.; NÄSSEL, D. R. (2000) Peptide-induced Ca^{2+} movements in a tonic insect muscle: effects of proctolin and periviscerokinin-2. **J. Neurophysiol.** 84, 3056-3075.

YASUHARA, T.; MANTEL, P.; NAKAJIMA, T.; PIEK, T. (1987) Two kinins isolated from an extract of the venom reservoirs of the solitary wasp *Megascolia flavifrons.* **Toxicon** 25, 527-535.

YOSHIDA, H.; GELLER, R. G.; PISANO, J. J. (1976) Vespulakinin: new carbohydrate-containing bradykinin derivates. **Biochemistry**.15, 61-64.

ZORNIK, E.; PAISLEY, K.; NICHOLS, R. (1999) Neuronal transmitters and a peptide modulate *Drosophila* heart rate. **Peptides** 20, 45-51.