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UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

INSTITUTO DE BIOTECNOLOGIA

Componentes del veneno del alacrán *Pandinus imperator* con actividad enzimática, antimicrobiana y antiparasitaria

T E S I S

Que para obtener el grado de

DOCTOR EN BIOTECNOLOGIA

p r e s e n t a

RENAUD CONDE BAEYE

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Tesis Doctoral.

"Componentes del veneno del alacrán *Pandinus imperator* con actividad enzimática, anti-microbiana y anti-parasitaria".

que para obtener el grado de DOCTOR EN BIOTECNOLOGÍA presenta

Renaud Condé Baeye.

Este trabajo fue realizado en el laboratorio del Dr. Lourival D. Possani, del Departamento de Reconocimiento Molecular y Bioestructura del Instituto de Biotecnología/UNAM.

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"A long push. a strong push. a push alltogether..." The Peter Principle (Peter Laurence Jhonson . 1969).

ÍNDICE

RESUMEN.

En esta tesis se presentan los resultados de la purificación y la caracterización de un péptido antimicrobiano llamado Escorpina, purificado del veneno del alacrán africano *Pandinus imperator*, así como la clonación del ADNc del gen que codifica para este péptido. Además se describe una nueva fosfolipasa A2 extraída del mismo veneno, así como la clonación del gen que codifica para ella. Ambos péptidos poseen características específicas que los hacen únicos en su clase; la Fosfolipina por ser una fosfolipasa A2 heterodimérica con semejanza estructural con las fosfolipasas A2 de abeja y *Heloderma*, y la Escorpina por ser un péptido que comparte características estructurales con dos familias distintas de péptidos antibióticos; las defensinas y las cecropinas.

ABSTRACT.

In this thesis I present the results of the isolation and characterization of an antimicrobial peptide called Scorpine purified from the venom of the African scorpion *Pandinus imperator*, as well as the cloning of a cDNA coding for this peptide. Additionaly, a novel phospholipase A2 extracted from the same venom is described, as well as the cloning of its encoding gene. Both peptides possess peculiar features that makes them unique in their class. The phospholipin for beeing an heterodimeric PLA2 with structural similarity to the honeybee and bead lizard PLA2 and Scorpine for beeing an antibiotic that shares structural similarity with two distinct antibiotic peptide families; the defensin and the cecropin peptides.

Lista de abreviaturas

µ:micro.
ADNc: Ácido DesoxiriboNucleotídico complementario.
AFP: anti fungal protein.
Dres: doctores.
fig.:figura.
FLA2: fosfolipasa A2.
g:grammos.
GIP: Gastric Inhibitory Polypeptide
Kd: constante de disociación.
ln: logaritmo neperianio.
LPS:lipo-polisacáridos.
m: mili
M:molar.
MIC: minimal inhibitory concentration.
mV: mili-Voltios.
NMR: Resonancia magnetica nuclear.
pH: potencial H+.
St.: Santo.

INTRODUCCIÓN.

I. Alacranes

I.1 Biología

Los alacranes pertenecen a uno de los órdenes de artrópodos terrestres más antiguos que se conocen, del que existen registros fósiles que datan del período Silúrico, esto es, de aproximadamente 400 millones de años atrás (Kjellesville-Waering 1986). Del punto de vista taxonómico los alacranes pertenecen al Reino Animalia (Subreino Metazoa), Phylum Arthropoda (Subphylum Chelicerata), Clase Arachnidae y al Orden Scorpionidae. Se reconocen 6 familias, a saber: Buthidae, Bothriuridae, Chactidae, Diplocentridae, Scorpionidae y Vejovidae. Todas las especies peligrosas al humano pertenecen a la familia Buthidae, de los géneros *Androctonus* sp., *Buthus* sp., *Leiurus* sp., *Mesobuthus* sp. y *Parabuthus* sp., en África y Medio Oriente, *Centruroides* sp., en México y el sur de Estados Unidos, y *Tityus* sp. en América del Sur (Keegan H.L., 1980). Si bien es cierto que este tipo de organismos, en términos de población, no representan una proporción importante del mundo animal, éstos son de importancia médica por la peligrosidad de su picadura.

I.2 Toxinas de alacranes

El componente principal del veneno de los alacranes lo constituyen las toxinas, éstas interactuan con canales iónicos de la membrana celular; también se encuentran algunas enzimas y moléculas de bajo peso molecular (Possani L.D., 1984). Las toxinas son una de las herramientas más utilizadas en el estudio de canales iónicos (Miller H. et al., 1995); dependiendo del tipo de canal que afectan, se reconocen como específicas para canales de potasio (Carbone E. et al., 1982), de sodio (Jover E. et al., 1980), cloro (DeBin J.A. et al., 1993) o calcio (Valdivia H., Possani L.D. 1998). Todas, salvo las específicas para calcio, adoptan un motivo estructural invariable conocido como α-ß estabilizado por cisteínas (Fontecilla-Camps J.C. et al., 1980; Bontems F. et al., 1991; Lippens W. et al., 1995; Delepierre M. et al., 1997; Oren Z. et al., 1998), el cual comparten con las defensinas de insecto y plantas, las tioninas gama y la brazeína.

I.3 Péptidos anti-microbianos identificados en los alacranes.

Es posible que la sobrevivencia de los alacranes como especie, se deba, al menos en parte, a la eficacia de su sistema inmune (Briggs D. E. G., 1987). En los alacranes se han encontrado distintos péptidos anti-microbianos (**figura 1**) tales como defensinas en la hemolinfa de *Leiurus quinquestriatus* (Cociancich S. et al., 1993) y *Androctonus australis* (Ehret-Sabatier L. et al., 1996); la androctonina, un péptido anti-microbiano que pertenece a la familia estructural de las tachyplesinas (Hetru C. et al., 2000); la Butinina, un péptido anti-microbiano de alacranes que está relacionado con las toxinas bloqueadoras de canales de potasio de alacranes; y la Hadrurina, un péptido que pertenece a la familia de los péptidos alfa-helicoidales. La Hadrurina fue encontrada en el veneno del alacrán, mientras que los demás péptidos antimicrobianos fueron encontrados en la hemolinfa de los alacranes (Torres-

Larios A. et al., 2000).

Hadrurina	**GILDTIKSIASKVWNSKTVQDLKRKGINWVANKLGVSPQAA**
Def *Lq*	**GFGCPLNQGACHRHCRSIRRRGGYCAGFFKQTCTCYRN**
Def4 *And au*	**GFGCPFNQGACHRHCRSIRRRGGYCAGLFKQTCTCYR**
Butinina	**SIVPIRCRSNRDCRRFCGFRGGRC.TYARQ.CLCGY**
Androctonina	**RSVCRQIKICRRR....GG.CYY.KCTNRPY**

Figura 1. Péptidos anti-microbianos de alacrán. Hadrurina; péptido anti-microbiano anfipático de hélice tipo alfa obtenido a partir de *Hadrurus aztecus* (Torres-Larios A. et al., 2000), Def *Lq*; defensina de *Leiurus quinquestratus* (Cociancich S. et al., 1993). Def4 *And au*; defensina 4 de *Androctonus australis*. (Ehret-Sabatier et al., 1996), Butinina; péptido anti-microbiano de *Androctonus australis* (Ehret-Sabatier et al., 1996). Androctonina; péptido tipo tachyplesina de *Androctonus australis* (Hetru C. et al., 2000).

Los péptidos anti-microbianos aislados a partir de distintos alacranes tienen estructuras primarias distintas a las estructuras reportadas para los péptidos anti-microbianos obtenidos a partir de otras fuentes. Las secuencias de las defensinas de alacranes se parecen solamente a la defensina de *Aeschna cyanea,* un insecto que pertenece al orden de los paleópteros, y la androctonina a las tachyplesinas de *Thachypleus tridentatus*, que comúnmente se considera como un fósil viviente. Por lo anterior, es posible que a partir del estudio de la genética y de las proteinas de estos organismos obtengamos información interesante sobre el proceso de evolución del sistema de inmunidad innata de los artrópodos en general, así como nuevos patrones estructurales de péptidos anti-microbianos.

I.4 *Pandinus imperator*

El *Pandinus imperator* (**Figura 2**) es el alacrán más grande que existe; pertenece a la familia de los *Scorpionidae*. *Pandinus* es el subgénero. Existen 24 especies de *Pandinus* descritas, todas de tamaño relativamente importante; *imperator* es la sub-especie. La primera descripción y cita de esta sub-especie es de Koch, C. L. (Koch C L., 1842.). Su distribución se limita básicamente a Africa central del Oeste (**Figura 3**).

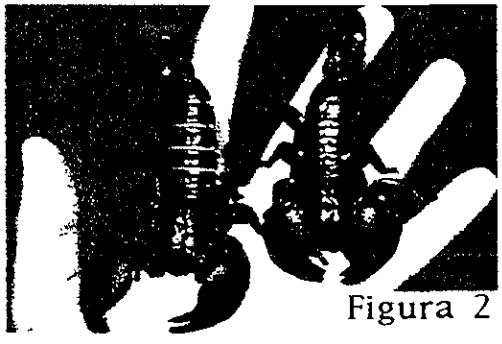

Figura 2

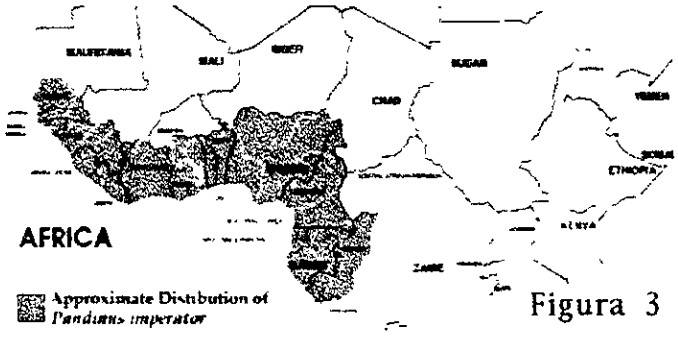

Figura 3

La picadura de esta especie de alacrán no es tóxica para los humanos. Se han extraido de su veneno diversos péptidos toxinas que activan el canal de calcio sensible a rianodina (Zamudio F. et al., 1997), toxinas bloqueadoras de canal de potasio (Olamendi-Portugal T et al , 1998), y dos fosfolipasas Estos péptidos podrian ser los responsables del dolor que se resiente después de una picadura (Zamudio F et al , 1997)

II. Péptidos anti-microbianos.

II.1 Generalidades.

Todos los organismos superiores presentan sistemas de defensa de tipo no-adaptativo contra las invasiones patogénicas (Medzhitov R. y Janeway R.A., 1997). Una parte importante de este sistema de defensa está constituído por la producción de péptidos que poseen función microbicida.

Las reacciones inmunes de tipo no–adaptativo representan a las primeras líneas de defensa en todos los organismos superiores, exceptuando a los vertebrados *gnasthostomas* que además presentan un sistema inmune de tipo adaptativo. El sistema innato de repuesta inmune está constituído por varios componentes: péptidos anti-microbianos, reacciones de fagocitosis, inhibición de proteasas y reacción de encapsulación por melanización. La respuesta inmune innata, si bien no es específica como la respuesta llevada a cabo por el sistema de anticuerpos, tiene la ventaja de ser energéticamente menos costosa (hasta 100 veces menor) y más rápida en llevarse a cabo (Barra D. et al., 1998). Los péptidos anti-microbianos son detectados en la hemolinfa de los insectos de 2 a 4 horas después de un daño séptico. Este tiempo de reacción es muy corto si se compara con el tiempo de la aparición de la respuesta inmune de tipo adaptativo (Meister et al., 1997).

La mayoría de los péptidos anti-microbianos han sido encontrados a través del estudio de la respuesta inmune innata no adaptativa, la cual aparece como el sistema de defensa primogénito en la evolución. Los insectos y en general los artrópodos, son conocidos por su resistencia a las infecciones bacterianas. Hace más de un siglo, Kowalesky (Kowalesky A., 1887) en St. Petersburgo y Cuenot en Nancy (Cuenot L., 1896) reportaron las reacciones inmunes en los insectos. Después de la Primera Guerra Mundial, Glaser, en Munich (Glaser R.W., 1918), Paillot, en Lyon (Paillot A., 1920) y Metalnikow, en París (Metalnikow S., 1920), demostraron que la reacción de defensa no se debía únicamente al proceso de fagocitosis de los microorganismos, sino también a la aparición de substancias bacteriolíticas en la hemolinfa de los insectos.

De manera sorprendente no fue hasta 1981 cuando se concretó, por el grupo de Boman y colaboradores, la identificación del primer péptido de insecto responsable de una actividad bactericida (Steiner H. et al., 1981). Desde entonces se han reportado un número creciente de péptidos en todos los *Phyla* de organismos, desde unicelulares hasta pluricelulares y desde procariontes a eucariontes.

Los pasados 50 años han constituído la era de los antibióticos químicos. Este período se caracterizó por el éxito encontrado en el uso de antibióticos no peptídicos (ya sean de origen natural o sintético) para combatir distintos tipos de infecciones provocadas por microrganismos. Sin embargo, actualmente los antibióticos convencionales están disminuyendo su eficacia (Travis J., 1994). Los niveles de resistencia a los antibióticos

"clásicos" por parte de los microorganismos se ha incrementado de manera preocupante en los últimos 20 años, y se pronostica que el ritmo de adquisición de resistencia se incrementará en los próximos años (Hancock R.E.W., 1997). De hecho, existen en la actualidad cepas de bacterias resistentes a todos los antibióticos disponibles (John C. C., 1999). Considerando este punto, los microorganismos generan poca resistencia a los péptidos anti-microbianos, razón por la cual éstos pudieran representar una alternativa para el control de los microorganismos patógenos (Saberwal G., Nagaraj R., 1994).

El bajo nivel de resistencia a los péptidos anti-microbianos por parte de los microorganismos se debe a que, en general, el blanco de éstos es la membrana lipídica, la cual resulta difícil de modificar; además, el efecto de los péptidos se da, de manera general, en un tiempo que cae en el orden de minutos, mientras que los antibióticos "clásicos" requieren de varias horas para eliminar a los microorganismos. Esta rapidez de acción deja poca oportunidad a los microrganismos para generar una mutación que le provea de resistencia (Zsaloff M., 1987).

Las posibles aplicaciones terapéuticas de diversos péptidos anti-microbianos han sido previamente evaluadas (Hancock R.E.W. and Lehrer R., 1998). Por el hecho de que las magaininas, cecropinas, defensinas y tachyplesinas ejercen su actividad directamente las membranas de los microorganismosm lo que es distinto al modo de acción de los antibióticos "clásicos" (por ejemplo la penicilina, la cefalosporina, la rifampicina, etc., los cuales interfieren ya sea sobre la síntesis de la pared celular o bien sobre la producción de proteínas de los microorganismos (Silver, L.L. and Bostian K.A., 1993), este tipo de péptidos se revelaron como buenos candidatos para aplicaciones médicas futuras. Cabe mencionar que, además de presentar actividad contra organismos patógenos, algunos de estos péptidos eliminan también a ciertas células cancerígenas (Anderson M. et al., 1995).

II.2 Familias estructurales de los péptidos anti-microbianos.

II.2.a Descripción general.

La gran variedad de los péptidos anti–microbianos encontrados en los últimos 20 años, y la poca información en cuanto a sus mecanismos moleculares de acción, han dificultado la realización de una clasificación sostenida y congruente. La clasificación estructural de este tipo de péptidos no tiene una correlación estricta con su modo de acción, ni con las posibles relaciones evolutivas que pudieran existir entre los diferentes integrantes de cada familia. Una propiedad importante en la mayoría de los péptidos anti-microbianos, es su capacidad en distinguir y eliminar de manera específica a los microorganismos blanco, sin eliminar a las células eucariontes hospederas. Quizá el único punto común, a nivel funcional, entre las diferentes clases de péptidos antibióticos es su afinidad por las membranas plasmáticas de los microorganismos. Este proceso se efectúa a través de una interacción específica con los fosfolípidos que la componen. Después de esta interacción, el efecto tóxico puede ser generado mediante varios mecanismos moleculares, tales como la

interacción con el ADN, el ARN, las proteínas intracelulares o de membrana y la destrucción de la integridad de la membrana citoplásmica del microrganismo blanco. La mayoría de los estudios llevados a cabo al respecto, apuntan a la importancia de la presencia de un carácter anfipático y de una carga neta positiva en estos péptidos (Blondelle S., Lhoner K., Aguilar M.A., 1999).

En 1985, A. Gururaj Rao clasificó a los péptidos anti-microbianos en 2 grandes familias: los péptidos lineales y los péptidos que presentan puentes disulfuro (Gururaj Rao A., 1985). En 1997, Robert Hancock incluyó a una tercera y cuarta familias dentro de esta clasificación, conformadas por los péptidos de hélice extendida y por los péptidos que forman asas gracias a la presencia de un puente disulfuro o de una ciclización covalente entre los extremos amino terminal y carboxilo terminal (Hancock R. E.W., 1997).

Los artrópodos y en particular los insectos, presentan en su sistema inmune un gran abanico de péptidos anti-microbianos con estructuras distintas (Meister M. et al., 1997). Es importante mencionar que se ha demostrado un efecto sinérgico entre ellos, aún cuando pertenezcan a clases estructurales distintas. Algunos ejemplos son los efectos sinérgicos notados entre la lebocina y la cecropina D (Hara S., Yamakawa M., 1995 (1)), entre la sarcotoxina IA y la proteína fungicida (o AFP) en *Sarcophaga peregrina* (Iijima R. et al., 1993) ó entre las magaininas PGLa y la magainina 2 (Westerhoff H.V. et al., 1995). También existen efectos sinérgicos con enzimas, como el efecto adyuvante de fosfolipasas tipo A2 (Buckland A.G., 2000).

El constante descubrimiento de péptidos anti–microbianos que presentan diferencias marcadas de secuencias dificulta el establecimiento de una clasificación coherente que se fundamente únicamente en los datos relativos a su estructura primaria (Tailor R.H. et al., 1997). Por otra parte, los datos de su estructura secundaria revelan conformaciones muy diversas: existen péptidos anti-microbianos con estructura de hoja beta plegada (como las defensinas de mamíferos), de estructura alfa hélice/beta plegada que se encuentra estabilizada por puentes disulfuro (como las defensinas de insectos), de pasador beta (como la tachyplesina), de alfa hélice con diferentes grados de anfipatía (como las cecropinas), de estructura desorganizada (como los péptidos ricos en prolina y en glicina), y de estructura de gancho, como la brevinina (Simmaco S. et al., 1993), entre otros.

Aunado a lo anterior, el estudio de la relación estructura-función de los péptidos anti-microbianos se complica por el hecho de que parecen ocurrir cambios a nivel de estructura secundaria cuando estos péptidos entran en contacto con la membrana celular de los microorganismos (Wei W. et al., 1998). El estudio más completo al respecto es el llevado a cabo sobre la inserción de la magainina (Wieprecht T. et al., 1999; Wenk R., Seeling J., 1998), el cual sugiere que la interacción entre el péptido y la membrana plasmática provoca un cambio estructural y termodinámico en ambos, lo cual favorece al estado de asociación frente al de disociación (para la defensina de *Allomyrina dichotoma*: consultar a Saido-Sakanaka H. et al., 1999; para la protegrina A, consultar a Rouemestand C. et al., 1998).

A la fecha, se han establecido tentativamente las siguientes categorías de péptidos anti-microbianos:

Familia	Características	Ejemplo	referencia
hélice alfa	Péptidos lineales con estructura de hélice alfa anfipática.	Cecropinas.	Holak T.A. et al..1988.
Beta Plegada	Péptidos de hoja beta plegada que se encuentra estabilizada por puentes disulfuro (1,2,3 o 4).	Defensinas, tachiplesinas...	Pardi A. et al., 1988.
Defensina de insectos	Péptidos de hélice alfa/hoja beta plegada que se encuentra estabilizada por puentes disulfuro (3)	Defensina A.	Dimarcq J L., 1998.
Péptidos lineales ricos en glicina	Péptidos que contienen más de 20% de glicinas en su secuencia peptídica y carecen de puentes disulfuros.	atacina de *Bombyx Mori*	Sugiyama M., et al., 1995.
Péptidos lineales ricos en triptófano	Péptidos lineales que contienen más de 15 % de residuos de triptófano en su secuencia y carecen de puentes disulfuros.	indolicidina	Subbalakshmi C., Sitaran N., 1998.
Péptidos cíclicos	Péptidos que forman un enlace entre su extremo amino terminal y carboxilo terminal..	circulina A	Tam J-P et al., 1999
Péptidos con amino acidos transformados.	Péptidos con aminoácidos no–naturales, glico–péptidos o lipo–péptidos .	Echinocandin FK463 (lipo-péptido)	Ito M. et al., 2000.
Péptidos *Proline Rich* (PR).	Péptidos lineales ricos en prolina y en arginina .	lobocina.	Hara S. et al., 1995.

El péptido antimicrobiano tratado en esta tésis participa de dos familias de péptidos antimicrobianos: los péptidos lineales anfipáticos y las defensinas de insectos.

Por esta razón la introducción que sigue tratará exclusivamente a dos familias de péptidos anti–microbianos: los péptidos lineales anfipáticos y los péptidos que presentan puentes disulfuros; con énfasis en las defensinas de insectos.

II.2.b Péptidos lineales anfipáticos.

Este tipo de péptidos fue descrito por primera vez en insectos en diapausa (Steiner H. et al., 1981). También están presentes en los mamíferos (Lee J.-Y. et al., 1989).

Estructura.

Las cecropinas de insectos están constituidas por alrededor de 25 aminoácidos y carecen de residuos de cisteína. Presentan una estructura de hélice alfa/conexión flexible/hélice alfa. Sin embargo, el número de aminoácidos que componen a los péptidos antimicrobianos lineales anfipáticos puede variar de 57 a.a. para la estielina C de *Styela clava* (Zhao C. et al., 1997) hasta 10 en los péptidos diseñados por Hong y colaboradores (Hong S. Y. et al., 1998). En el caso de las cecropinas canónicas (la sarcotoxina IA (Iwai H. et al., 1993) y la cecropina A (T.A. Holak et al., 1988)), la hélice alfa amino terminal es de carácter anfipático, mientras que la hélice alfa carboxilo terminal es más hidrofóbica

En un medio acuoso este tipo de péptidos carecen de una estructura secundaria bien definida (Wang W., 1999). Sin embargo, se estructuran en hélice alfa tras su contacto con liposomas o con solventes orgánicos polares (Wang W. et al., 1998). Se ha propuesto que su interacción con la membrana lipídica induce cambios conformacionales que incrementan su estructuración en hélice alfa (Blondelle S. et al., 1999).

Figura 4. Representación de la estructura tridimensional de la cecropina P1 en solución de agua con 30 % de 1,1,1,3,3,3 exafluoro 2 propanol deducida a partir de datos de Resonancia Magnética Nuclear (o NMR por sus iniciales en inglés) (Sipos D., Andersson M., Ehrenberg A., 1992). En azul se encuentran representados los aminoácidos hidrofóbicos; en verde, los aminoácidos cargados positivos en agua a pH 7.0.

En el **I** se presenta un alineamiento de secuencias de temporinas y magaininas y en el **Anexo II** se presenta un alineamiento de cecropinas; todos péptidos que pertenecen a esta clase estructural.

Actividades y posible mecanismos de acción de los péptidos lineales anfipáticos.

Los péptidos lineales anfipáticos actúan a nivel de la membrana de los microorganismos. Su modo de acción radica en la desestabilización de la membrana mediante la formación de poros iónicos transientes (Merrifield R. B. et al., 1994). La aparición de poros iónicos o de actividad semejante a poros iónicos, ha sido reportado para las magaininas en el sistema de bicapas lipídicas planas (Cruciani R. A. et al., 1991).

En los procariontes, la cascada de fosforilación oxidativa se realiza a través de bombas de protones que se localizan en la membrana citoplásmica (Bechinger B., 1997). Por ello, el efecto tóxico de la formación de poros se debe a la disipación del gradiente iónico que rige su estado metabólico energético.

A través del transporte de electrones o a través de la hidrólisis de ATP, las bacterias mantienen su potencial de membrana. Mediante la expulsión continua de protones mantienen a una fuerza de transporte que incluye un gradiente de potencial ($\Delta\Psi$ que está orientado con el lado interno negativo) y a un gradiente de pH (ΔpH, que presenta al pH más alcalino interno). A pH neutro, la principal fuerza motriz está dada por el gradiente de potencial (que es de -150 mV), el cual se mantiene por los flujos de K+ (Wu M. et al., 1999). Se ha propuesto que la composición diferente en fosfolípidos carcados negativamente de las membranas bacterianas así como la elevada diferencia de potencial a través de éstas, modulan la afinidad específica de estos péptidos (revisado en Bechinger B., 1997).

En contraste, los péptidos alfa helicoidales líticos que actúan sobre organismos eucariontes se regulan por otro tipo de factores. Por una parte, es necesaria una mayor afinidad del péptido por la parte hidrofóbica de la bicapa para integrarse en la membrana, la cual generalmente para organismos eucariontes está compuesta por fosfolípidos neutros. Por otra parte, ya que las células eucariontes tienen la posibilidad de regular su volumen a través de bombas iónicas protéicas, la fuga de iones provocada por los péptidos líticos debe sobrepasar la acción de estas bombas para tener efecto (Thennarasu S., Nagaraj R., 1995).

Se ha intentado relacionar la capacidad de los péptidos antimicrobianos para matar a los microorganismos, por formación de poros en su membrana, mediante el análisis de sus características estructurales,. El estudio matemático más avanzado es el reportado por Pathak N. y colaboradores (1995). La fórmula propuesta por estos autores es la siguiente:

$$\ln(1/MIC + 0.015)=3.023\ H + 9.551M + 0.201\alpha - 7.462$$

donde H es la hidrofobicidad media del péptido, M es el momento hidrofóbico, α es el contenido de hélice alfa en el péptido, y MIC es la concentración inhibitoria mínima contra *Escherichia coli.*

La formación de poros se ha explicado por varias teorías representadas en la **Figura 5**. La primer teoría (A) se refiere a la agregación de los peptidos en complejos transmembranales que son perpendiculares al plano de la membrana, los cuales forman poros semejantes a los poros de los canales iónicos. El centro del poro del canal es de naturaleza protéica. Otra teoría (B) propone que la formación de poros se debe a la inserción del conjunto de péptidos en la región hidrofóbica de la membrana, que provoca una curvatura en ésta. El centro del poro del canal, en este caso, está conformado por las cabezas hidrofílicas de los fosfolípidos. Un tercer modelo (C) describe a los péptidos situados en paralelo al plan de la membrana lipídica, los cuales crean una brecha transitoria o "defecto" en la curvatura de la membrana de los microorganismos que permite el paso de iones y de metabolitos durante el lapso de tiempo en el que la brecha se abre (*carpet like*). Un cuarto modelo (D) es el del "efecto detergente", que resulta de una acumulación de los péptidos en la superficie de la membrana seguida por una solubilización de la membrana a través de la formación de micelas. La quinta teoría (E) está sustentada por el modelo de difusión de los péptidos en el plano de la bicapa, que provoca un desarreglo local en la membrana. Los péptidos al difundirse en ésta, se podrían acercar brevemente, sobrelapando las áreas de desarreglo, dejando así una brecha transitoria en la membrana. Este modelo se propone para las cecropinas y las magaininas y podría explicar la disposición de los péptidos en paralelo al plano de la membrana.

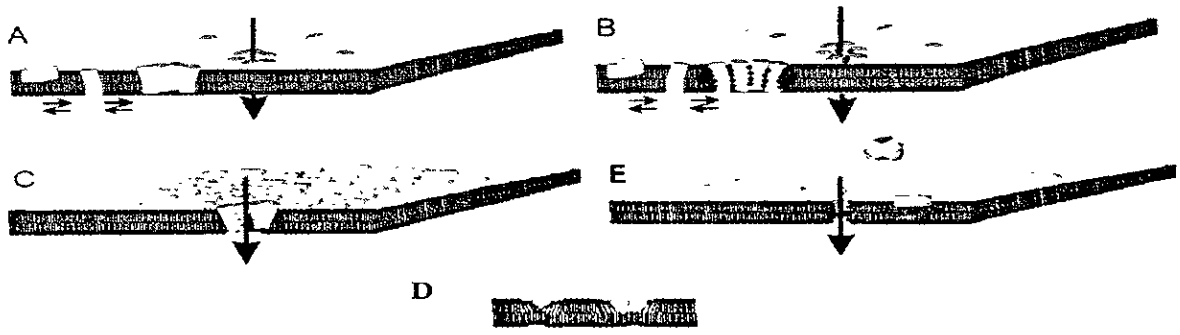

Figura 5. Modelos de formación de poros por los péptidos anti-microbianos anfipaticos. A: Poro peptídico; B: Poro cubierto por las cabezas de los fosfolípidos; C: Efecto de tapete o *carpet-like*; D: "Efecto detergente"; E: Difusión en el plano de la membrana. Cada uno de estos modelos se ha generado a partir de algún péptido tipo, sobre el cual se ha podido demostrar que éste es el funcionamiento que explica su actividad anti-microbiana (Revisado en : Bechinger B., 1999).

Mecanismos de inhibición que no involucran perturbación de la membrana plasmática han sido encontrados con péptidos anti-microbianos de esta misma clase estructural. Por ejemplo, la buforina II mata a los microorganismos al internalizarse en éstos. Este péptido se acumula y se liga a los ácidos nucleícos y de esta manera inhibe la síntesis protéica en el organismo blanco (*E. coli*). Sin embargo, la buforina II es también capaz de formar poros dependientes de voltaje en membranas artificiales (Park C.B. et al., 1998).

II.2.c Defensinas.

Dentro de esta clase estructural de péptidos anti-microbianos se incluyen a 4 subclases; las defensinas de insectos, las defensinas de plantas (o beta-tioninas), y las defensinas alfa y beta de mamíferos. Se trata de péptidos constituidos por alrededor de 35 aminoácidos, el número de puentes disulfuro presentes en éstos es de tres a cuatro. La conectividad de las cisteínas en estas cuatro familias de defensinas es distinta entre sí, sin embargo; su estructura tiene un punto común: la presencia de una hoja beta plegada que está estabilizada a través de puentes disulfuro (Bonmatin J.M. et al., 1992).

Clases estructurales.

Defensinas de insectos

Las defensinas de insectos (llamadas también defensinas de invertebrados) tienen una conectividad de puentes disulfuro 1-4, 2-5, 3-6. El puente disulfuro establecido entre la primera y la cuarta cisteína mantienen al asa N-terminal, mientras que el resto de los puentes disulfuro conectan a la hoja beta plegada con la hélice anfipática (Dimarcq Jean-Luc et al., 1998). Las defensinas de insectos tienen la peculiaridad de presentar una hélice alfa, además de la hoja beta plegada que es característica en las alfa y beta defensinas. La hélice alfa mantiene a la hoja beta plegada mediante puentes disulfuro, generando una estructura de nudo beta-alfa-beta, que es el mismo esquema estructural que se encuentra en las toxinas de alacranes que bloquean a los canales de potasio. De hecho, se reportó que la sapecina B de *Sarcophaga peregrina* es capaz de inhibir al canal de potasio de rata que se activa por calcio con una Kd cercana a la Kd de la caribdotoxina (Shimoda M. et. al., 1994). En la tabla incluida en el **Anexo III** se presenta un alineamiento de distintas defensinas de artrópodos.

Figura 6: Modelo de la defensina A de *Protophormia terraenovae* (Cornet B. et al., 1995)

Defensinas de plantas.

Las defensinas de plantas presentan entre 3 y 4 puentes disulfuro y tienen como motivo estructural común a una triple hoja beta antiparalela torcida que está estabilizada por los puentes disulfuro. La conectividad de las cisteínas en los péptidos es de 1-4, 2-5, 3-6 para las defensinas que presentan 3 puentes disulfuro y de 1-4, 2-5, 3-6, 7-8 para las defensinas que contienen a cuatro puentes disulfuro (Martins J.C. et al., 1996). Las defensinas de plantas con tres puentes disulfuro, como la proteína anti-microbiana 2 de *Amaranthus caudatus* (que se une específicamente a azucares de la membrana de los microorganismos blanco), se asemejan estructuralmente a las agatoxinas (Cammue B.P.A., 1991). En general, este tipo de péptidos poseen actividad contra hongos, aunque también presentan una actividad menor contra bacterias Gram-negativas o Gram-positivas.

Existen también defensinas en plantas que poseen a cuatro puentes disulfuro con una conectividad de 1-8, 2-5, 3-6, 4-7, y son llamadas gama-tioninas. Estas tienen una estructura semejante a las toxinas de alacranes: presentan además de una triple hoja beta, a una hélice alfa que está estabilizada por puentes disulfuro. Son activas contra bacterias y contra hongos (Bloch Jr. C. et al., 1998). Es notable que la drosomicina que se obtiene a partir de *Drosophila melanogaster* presente cuatro puentes disulfuro así como una arquitectura semejante a las defensinas de plantas (Fhelbaum P. et al., 1994).

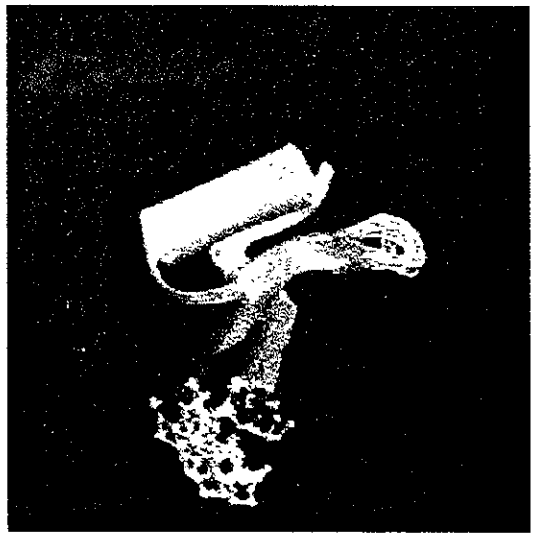

Figura 7. Estructura de la gama-tionina de rabano *Raphanus sativus* (Fant F. et al.,1998).

Defensinas tipo alfa y beta.

Las defensinas de mamíferos se caracterizan por estar constituidas por una hoja beta antiparalela ligeramente torcida que se mantiene por tres puentes disulfuro (Pardi A. et al., 1988). La diferencia entre las defensinas de tipo alfa y beta radica en la conectividad de sus puentes disulfuro. Las defensinas de tipo alfa conectan a sus cisteínas en el orden 1-6, 2-4, 3-

5; mientras que las defensinas de tipo beta conectan a sus cisteínas en el orden 1-5, 2-4, 3-6 (Nicolas P., 1995). La tercera familia que se relaciona con las defensinas es propia del estómago y los intestinos, y son llamadas criptidinas. El apareamiento de los puentes disulfuro en éstas es 1-6, 2-4, 3-5. Sin embargo, el alineamiento de distintas secuencias revela diferencias estructurales con las defensinas de tipo alfa (Ouellette A. J., Lualdi J. C., 1990).

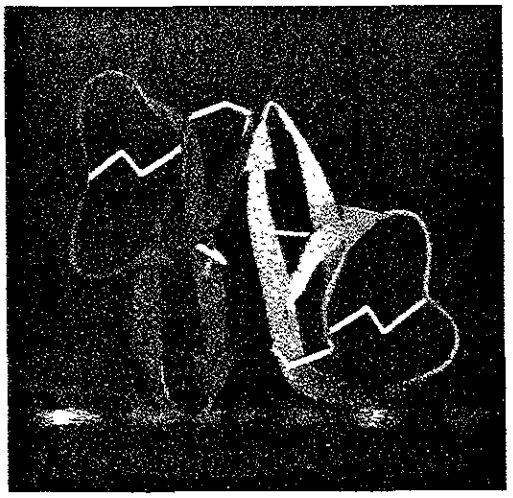

Figura 8: Modelo de la estructura de la defensina α H-NP3 de humano (Hill. C. P. et al., 1991).

Se propone que las defensinas de tipo beta fueron generadas antes que las defensinas de tipo alfa. De hecho, sólo las defensinas de tipo beta están presentes en aves (Harwig S. S. L. et al., 1994). Sin embargo, ambos tipos de defensinas comparten a un ancestro común (Liu L. et al., 1997).
La tabla incluida en el **Anexo IV** presenta un alineamiento de las defensinas de tipo alfa, beta y criptidinas.

Actividades y posible mecanismos de acción de las defensinas.

Contrariamente a lo que ocurre con los péptidos lineales, los péptidos que contienen puentes disulfuro presentan una estructura más rígida, lo cual permite (a partir de los datos obtenidos de estudios de cristalografía o de NMR) una predicción más precisa de su estructura secundaria. Mediante el uso de esta herramienta, se ha propuesto que la interacción entre la defensina Hs-AFP1 de *Heuchera sanguinea* (esto es, una defensina vegetal) con el microrganismo blanco se establece a través del reconocimiento de las proteínas membranales de éste último (Thevisen et al., 1997). Sin embargo, en general, el blanco inicial es la parte lipídica de la membrana, tal y como ocurre con los péptidos anfipáticos

Asimismo, se ha demostrado la capacidad de formación de poros transitorios en bicapas lipídicas planas por las defensinas. La defensina H-NP3 (de origen humano) forma poros dependientes de voltaje en las bicapas modelo, desde concentraciones de 0,3 μM (Wimley W.C et al , 1994). La propuesta de la estructura del poro formado por la H-NP3

está representada en la **Figura 9.**

En el caso de las defensinas de plantas, se ha descubierto que la proteína fungicida 2 de *Raphanus sativus* (Rs-AFP2) opera a través del reconocimiento de un receptor protéico que se localiza en la superficie de la membrana del organismo blanco (Thevissen K. et al., 1996). Considerando que el plegamiento de las defensinas es similar al plegamiento de toxinas que afectan canales de K^+ o de factores de regulación (uromoddulina, Factor H del complemento), es posible que las interacciones de las defensinas sean más complejas que una interracción con la parte lipídica de la membrana: involucrando proteínas membranales de los microrganismos, ó interaccionando con el ADN.

Las defensinas de insectos posiblemente actúen bajo el mismo principio deformación de poros discretos que las defensinas de mamíferos. El estudio de la defensina A de *Phormia terranovae* reveló mecanismos complejos de interacción con las membranas lipídicas. Se demostró que tras la unión de los péptidos a la membrana, los monómeros de la defensina A tienden a asociarse entre sí, formando multímeros. La especificidad de este tipo de péptidos radica en la afinidad de la defensina A por los fosfolípidos aniónicos saturados. Una vez que la defensina A de *Phormia terranovae* penetra profundamente en la membrana, la asociación de los monómeros entre sí genera brechas. La desestabilización de la membrana mediante este tipo de estructuras explica la aparición de actividades semejantes a poros iónicos en las membranas blanco (Maguet-Dana R., Ptak M., 1997). Por otro lado, la interacción del péptido con la membrana produce cambios estructurales en la defensina, que se traducen en cambios termodinámicos y, por ende, en el aumento de la afinidad de la defensina A por las membranas de los microorganismos (Maguet Dana R., 1995).

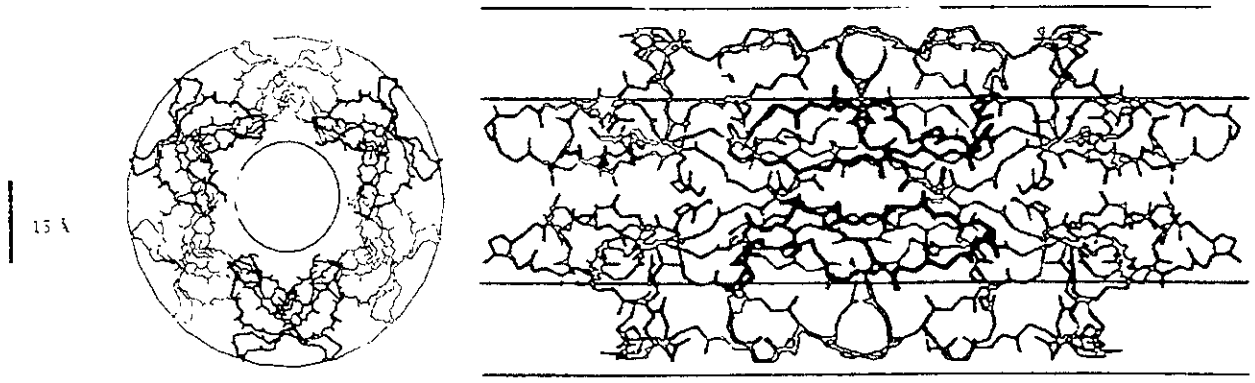

Figura 9. Modelo del poro formado por la defensina H-NP3 de *Homo sapiens sapiens*, en base a su estructura por critalografía (Wimley W.C. et al., 1994). Se presenta el poro formado por 6 dímeros de la misma defensina: los trazos marcados alternadamente negros y grises representan cada uno, un dímero de defensina HNP3.

II.2.d Otros péptidos que presentan puentes disulfuro.

Tachyplesinas y análogos. (hoja beta plegada)

Takanori Nakamura y colaboradores (1988) describieron a una nueva subfamilia de péptidos anti-microbianos que se definen por la presencia de una estructura de hoja beta que está estabilizada por solo dos puentes disulfuro. Se trata de las tachyplesinas y otros péptidos análogos, que fueron descubiertos en la hemolinfa del cangrejo *Thachypleus tridentatus*. Las tachyplesinas se caracterizan por ser mucho más cortas que las defensinas (presentan alrededor de 17 aminoácidos) y por formar solamente dos puentes disulfuro. Mandart y colaboradores (1999), encontraron un péptido anti–microbiano de esta clase en la hemolinfa del alacrán *Androctonus australis*. Los resultados obtenidos por NMR en medio acuoso muestran que la estructura secundaria presenta el aspecto de un pasador beta plegado antiparalelo torcido estabilizado por dos puentes disulfuro. Este péptido, en contacto con la membrana lipídica pierde un poco de su estructura de hoja beta plegada (Mandart N. et al., 1999).

Brevininas y análogos. (hoja beta plegada-estructura de gancho)

Esta familia incluye a los péptidos anti-microbianos más cortos, que se componen exclusivamente de 8 (bradikinin (Basir Y.J. et al., 2000)) a 32 aminoácidos (esculentina (Simmaco M. et al., 1993)). Presentan un asa de 5 residuos en su extremo carboxilo terminal, la cual se mantiene a través de un sólo puente disulfuro. Esta estructura se define como estructura de gancho C-terminal. Algunos miembros de esta familia presentan actividad hemolítica (como la brevinina 1E), mientras que otros no (como la esculentina), pero todos los péptidos que se encuentran incluidos en ella se caracterizan por poseer actividad anti-microbiana contra bacterias Gram-positivas y Gram-negativas a concentración micromolar (Simmaco M. et al., 1993; Morikawa N. et al., 1992).

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Brevinina 1E                          FLPLLAGLAANFLPKIFCKITRKC (R)
Brevinina 1                           FLPVLAGLAANFLPKIFCKITRKC (X)
Brevinina 2E                GIMDTLKNLAKTAGKGALQSLLNKASCKLSGQC (R)
Brevinina 2                 GLLDSLKGFAATAGKGVLQSLLSTASCKLAKTC (X)
Esculentina  GIFSKLGRKKIKNLLISGKNVGKEVGMDVVRTGIDIAGCKIKGEC (R)
```

Figura 10. Secuencia aminoacídica de distintas brevininas que se han purificado a partir de diversas fuentes. Las secuencias marcadas con (R) al final son de Simmaco M. et al., 1993 y las secuencias marcadas con (X) al final son de Morikawa N. et al., 1992

Actividades y posibles mecanismos de acción.

La mayoría de estos péptidos se unen a la membrana lipídica de los microorganismos, causando una perturbación en ésta que provoca una fuga de metabolitos y con ello, la destrucción del gradiente electroquímico.

De manera similar al resto de los péptidos anti-microbianos, la estructura secundaria de las tachyplesinas se modifica cuando el péptido entra en contacto con los fosfolípidos de

la membrana lipídica (Mandart et al.. 1999).

II.2.e Péptidos híbridos.

La comparación de la secuencia de aminoácidos de ciertos péptidos con la familias descritas en párrafos anteriores, indica la existencia de un tipo de péptidos que poseen motivos estructurales pertenecientes a distintas clases de péptidos anti-microbianos. Tal es el caso de la "big defensin", que se obtuvo a partir de hemocitos del cangrejo *Tachypleus tridentatus*. Este péptido presenta una estructura de defensina en su región carboxilo terminal, con una disposición de puentes disulfuro semejante a las defensinas tipo beta encontradas en mamíferos. Sin embargo, el extremo amino terminal forma una hélice alfa de carácter hidrofóbico, que provee a la molécula completa de una estructura anfipática. El péptido completo es activo contra *Salmonella typhymurium* (a una MIC -Concentración Mínima Inhibitoria- de 20 mg/mL) y contra *Staphylococcus aureus* (a una MIC de 2.5 mg/mL). Cuando se separan ambos extremos de la molécula. la región amino terminal retiene su actividad contra *Staphylococcus aureus* pero pierde su actividad contra *Salmonella typhymurium*. Lo contrario es cierto para la región carboxilo terminal. Sin embargo, para la función anti-LPS (función de pegado a los lipo-polisacaridos microbianos) se requiere de la molécula completa (Saito T. et al., 1995).

En el cerdo se presenta también el caso de péptidos precursores a los péptidos anti–microbianos que tienen en su versión completa una función distinta. La proteína *GIP* (por Gastric Inhibitory Polypeptide) cuenta con una actividad hormonal del tipo glucagón. pero la versión naturalmente procesada (que presenta una extensión de 7 a 42 aminoácidos) presenta actividad anti–microbiana contra *Bacillus megaterium* (Agerberh B. et al., 1993).

Las catelicidinas se procesan post-traduccionalmente en distintas clases de péptidos anti–microbianos (por ejemplo en péptidos ricos en prolinas, en péptidos alfa helicoidales anfipáticos y en péptidos ricos en cisteínas que son semejantes a las tachyplesinas) y se encuentran en varios grados de procesamiento en los leucocitos. Cada producto se caracteriza por una actividad anti-microbiana específica (Storici P. et al.. 1996).

II.2.f Péptidos antimicrobianos y malaria.

Una aplicación potencial de los péptidos antimicrobianos ha sido la búsqueda de nuevos antibióticos, que pudieran curar infecciones resistentes a los tratamientos por los antibióticos convencionales.

En el caso de la malaria, se investigaron el efecto plasmicido de distintos péptidos antimicrobianos. Se planteó el tratamiento del huésped humano del parásito con la dermaseptina 3, un péptido antimicrobiano de rana. Las dermaseptinas S3 y S4 fueron estudiadas y se demostró su capacidad de matar al *Plasmodium* en el eritrocito, así como su capacidad de lisis selectiva de los eritrocitos infectados (Krugliak M. et al., 2000).

También se planteó el uso mosquitos transgénicos que expresen péptidos antimicrobianos que impidan el establecimiento del parásito (James A.A. et al.. 1999).

III Malaria.

III.1 Generalidades.

La malaria es una enfermedad parasitaria causada por *Plasmodium sp.* que afecta a mamíferos, a reptiles y a aves. Se han identificado cuatro especies distintas de *Plasmodium* que infectan a los humanos: *P. falciparum*, *P. vivax*, *P. ovale* y *P. malariae*.

Este parásito se transmite por mosquitos pertenecientes al género *Anopheles*, que abunda en los climas tropicales, principalmente en Africa, en América Central y del Sur y en Asia. En este momento, al menos 300 millones de personas están afectadas por la malaria en el mundo (Gratz N., 1999), y se calcula que más de 2 millones de personas mueren a consecuencia de esta enfermedad cada año (White N.J. et al., 1999). La malaria es generalmente endémica en la zona de los trópicos y en las zonas subtropicales. En la **Figura 11** se indican las zonas de distribución de la malaria en 1990, según información obtenida de la Organización Mundial de la Salud (o WHO por sus iniciales en inglés). A la fecha, el 80 % de los casos reportados se localizaron en Africa, siendo *P. falciparum* la principal especie del parásito encontrada a nivel clínico. Las 3 especies restantes (*P. vivax*, *P. ovale* y *P. malariae*), si bien no representan una mortalidad importante por sí solas, pueden co-infectar a pacientes infectados por *P. falciparum*, aumentando de esta forma la complejidad del tratamiento.

Figura 11. Distribución geográfica de la malaria. Las zonas marcadas en negro representan a las zonas con mayor peligro de transmisión.

De acuerdo con la WHO (1999), el aumento reciente de la incidencia de la malaria se debe a diferentes factores, entre otros, a la mayor movilidad de las poblaciones, al decaimiento en las medidas de prevención en los países en vías de desarollo y a la aparición de cepas de *Plasmodium* resistentes a los tratamientos por cloroquinina.

A partir de la fecha en la cuál se detectó resistencia a las drogas anti-maláricas (en 1960), se inició una difusión global de las cepas resistentes. De manera simultánea, aparecieron formas más agresivas y mortales de *Plasmodium falciparum*. El número de casos

de malaria aumentó también debido a la aparición del transporte aéreo, que facilitó la difusión de nuevas cepas de *Plasmodium* en regiones en las cuales la malaria local estaba controlada (como en los Estados Unidos de Norte América). Los casos de malaria en poblaciones no inmunes, en general, provocaron la muerte de los pacientes. Este hecho apoya la hipótesis que propone que una globalización de la infección por *Plasmodium* tendría consecuencias importantes en las poblaciones que nunca han estado en contacto con el microorganismo (Kidson C. Indaratna K., 1995). Es notable que la erradicación de la malaria esté relacionada con procesos macroeconómicos que no están directamente involucrados con el sector salud, como el sistema de cultivo, la presencia de pantanos no secados, migraciones de refugiados que vienen de zonas endémicas de malaria, etc.. Sin embargo, los aspectos médicos de la enfermedad son los únicos factores que los países involucrados suelen considerar. Históricamente la malaria ha tenido un impacto negativo, severo y medible, sobre la productividad de las naciones con poblaciones que la padecen (Nabarro D. , 1999).

III.2 Biología del *Plasmodium.*

El ciclo de vida del microorganismo requiere de un vector para su dispersión: los mosquitos del género *Anopheles*. Si bien la malaria puede trasmitirse por transfusión sanguínea, en general, los mosquitos del género *Anopheles* son los principales vectores de la transmisión del *Plasmodium*. El número de vectores implicados en la transmisión de la malaria es muy alto. En 1993, fueron consideradas 51 especies distintas de *Anopheles* como relevantes en la transmisión del *Plasmodium* (Brown D.N., Nelson M., 1993). En México, los mosquitos vectores de la malaria son *A. pseudopunctipenis* y *A. albimanus* (Rodríguez M.H., Loyola E. G. 1989).
El ciclo de reproducción del *Plasmodium* está representado en la **Figura 12**.

En el mosquito: Los micro-gametocitos hembra y macho son trasmitidos mediante la toma de sangre por el mosquito, y se transforman en microgametos y macrogametos. La fertilización ocurre y los zigotos se transforman en oocinetos, que invaden el mosquito. Los oocinetos se transforman en ooquistes que se ubican en la pared celular del estómago, y son liberados en la hemolinfa, un liquido que llena las cavidades que rodean todos los órganos de los insectos. Los ooquistes se transforman en esporozoítos que migran hacia las glándulas salivales para infectar al huésped animal en la próxima toma de sangre. Para pasar a la glándula salival el esporozoíto necesita atravesar varias barreras: la lamina basal, las membranas plasmática basal y apical de las células secretorias, y la pared del ducto salival. *P. berghei* invade la región distal de los lóbulos de la glándula salivaria de *Anopheles stephensi*, rompe la membrana basal, la membrana plasmática de las células secretoras y se acumula en las cavidades salivales secretoras (Sterling C.R. et al., 1973).

Una vez inyectado por el mosquito hembra *Anopheles* en el huésped, el esporozoito entra en el torrente sanguíneo y alcanza los hepatocitos. Tras penetrarlos, forma los esquizontes, que liberan a los merozoítos en el torrente sanguíneo del huésped. Los merozoítos invaden a los eritrocitos, y se desarrollan sucesivamente en la forma de anillo,

trofozoítos y esquizontes (que constituyen la vía asexuada de multiplicación) o secuencialmente en forma de anillo y gametocitos (esto es, la vía sexuada de diferenciación) (Hadley T. J. et al., 1986).

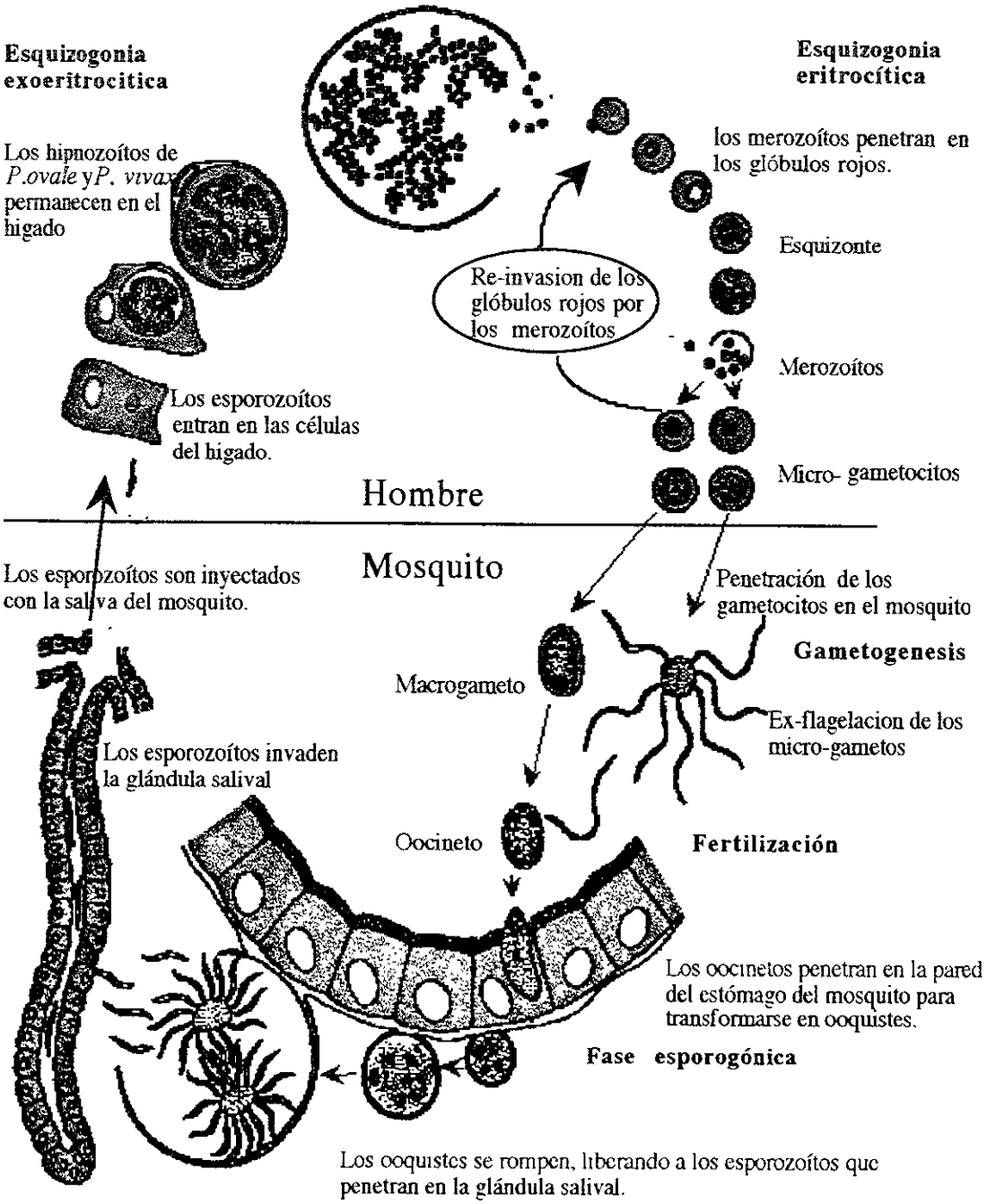

Figura 12. Ciclo de vida del *Plasmodium* (Bradley T., 1993)

Los pasos críticos para la supervivencia del parásito parecen darse durante la infección del estómago del mosquito, al cual el *Plasmodium* se ha vuelto sumamente especializado (Shahabuddin M., Kaslow D. C., 1994.). La fase de oocineto es particularmente sensible a los péptidos endógenos como las defensinas del mismo mosquito. Se sabe que el establecimiento del parásito en las paredes del estómago del mosquito es crucial para su desarrollo, y que esta fase activa las vías de síntesis de péptidos anti-microbianos endógenos del mosquito (Richman A.M. et al., 1997). En los mosquitos competentes para su trasmisión, la respuesta inmune generada por *Plasmodium* es más débil que la reacción inmune generada por una infección por bacteria; y no activa la producción de defensina (Lowenbergher C.A. et al., 1999).

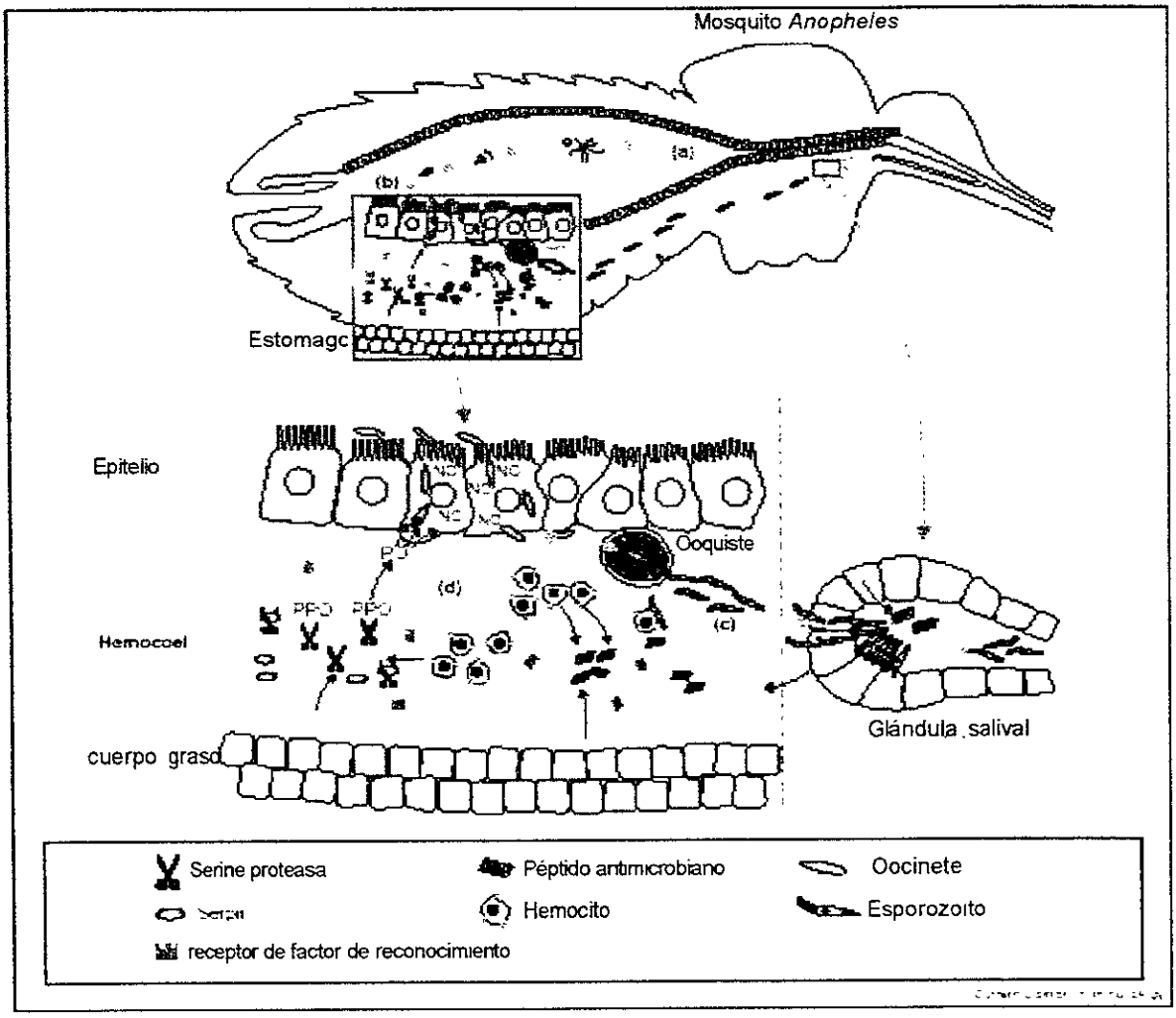

Figura 13 Modelo del ciclo de vida del *Plasmodium* en el mosquito vector, y de los mecanismos potenciales de defensa desarrollados contra la infección. La ruta de desarrollo del *Plasmodium* es enseñada por las flechas amarillas; las respuestas anti-*Plasmodium* son indicadas por las flechas rojas. Las flechas azules indican las zonas de ampliación del esquema general. (a) Los gametocitos ingeridos se fertilizan y forman un zigoto, que se transforma en un oocineto móvil. (b) El oocineto invade y atraviesa las células epiteliales del estómago, alcanzando la lámina basal, donde se transforma en ooquiste. (c) Los ooquistes se desarrollan y son liberados en el hemocoel, desde este pueden invadir las glándulas salivales

La respuesta inmune del mosquito contra el parásito ha sido reportada en el epitelio

del estómago, en el hemocoel y en las glándulas salivales. El ciclo de vida del parásito en el mosquito está representado en, la **figura 13.**

En el epitelio del estómago de mosquito resistente al *Plasmodium*, el parásito es encapsulado ó lisado. El NO (monóxido de nitrógeno) restringe el desarrollo del parásito en el estómago. En el hemocoel, los esporozoitos pueden encontrar hemocitos, péptidos antimicrobianos y otras secreciones del hemocoel. La unión del parásito a receptores de factores de reconocimiento del *Plasmodium* desencadena una cascada de reacciones mediada por serina-proteasas, que conduce en el corte de profenol oxidasa en fenol oxidasa, enzima involucrada en la encapsulación del parásito. Las serpinas inactivan las serina-proteasas. En la glándula salival también se encuentran péptidos antimicrobianos (Dimopoulos et al., 2001).

Estructura de la población de *Plasmodium*

El *Plasmodium* tal como lo conocemos parece haber evolucionado en conjunto con el hombre. Se ha inferido la presencia de un "cuello de botella" en la evolución de las poblaciones de *Plasmodium* por el hecho de que las poblaciones de *Plasmodium* actuales no se presentan muchas mutaciones silenciosas en los genomas, y se calcula que su ancestro común surgió hace aproximadamente 20.000 años (Rich S.M. et al., 1998). Los análisis filogenéticos de las distintas subespecies de *Plasmodium* realizado en base a las zonas 5' no codificantes del gene circumsporozoite generan el dendograma presentado en la **Figura 14**: en el se puede apreciar que las cuatro especies que afectan a los seres humanos están más cercanas a las especies de *Plasmodium* que afectan a otros mamíferos, reptiles y aves, que entre ellas mismas.

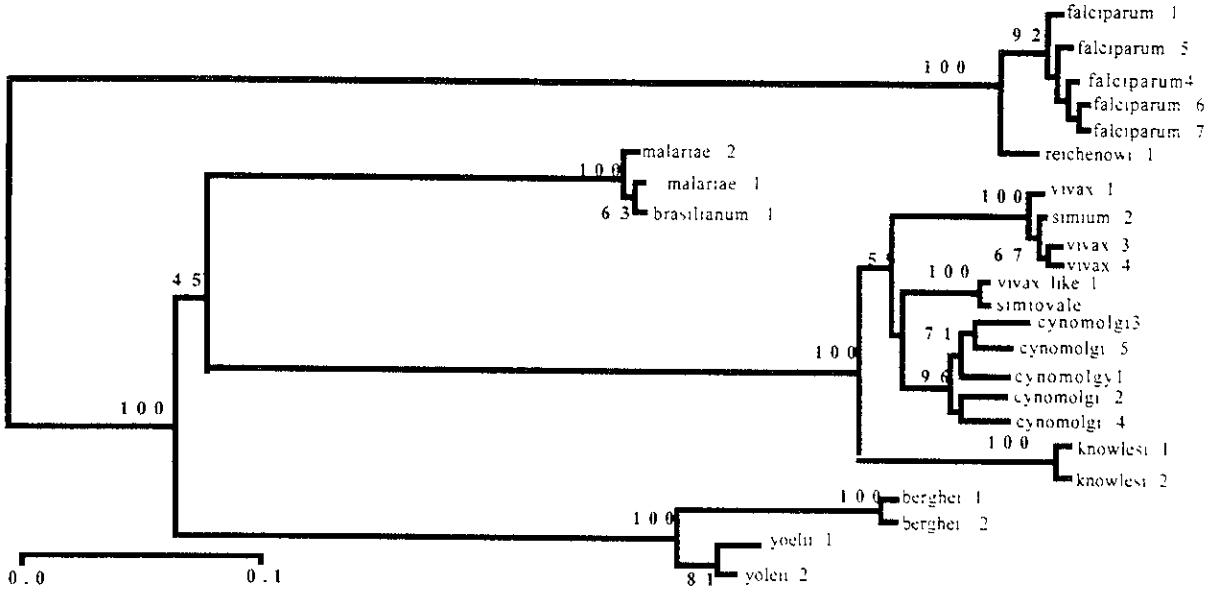

Figura 14. Filogenia de 12 especies distintas de *Plasmodium*. La relación fue inferida a partir de los genes *Csp* Los valores "Bootstrap" mayores de 70 son estadísticamente confiables (Ayala F. J. et al., 1999).

A pesar de un tiempo de evolución relativamente corto, el *Plasmodium* presenta una gran variabilidad de antígenos presentados en su superficie. Esto se debe a que

las proteínas de superficie del microorganismo presentan zonas de repeticiones que facilitan la aparición de mutaciones. Esta variabilidad en partes peculiar de su genoma le ha permitido evadir la presión inmunológica de sus hospederos a lo largo de su evolución (Rich S.M., Ayala F.J., 2000). Es importante mencionar que estas características han resultado sumamente inconvenientes para el desarollo de vacunas.

III.3 Control de la transmisión del *Plasmodium.*

Distintas vías para la reducción de la transmisión de la malaria se han explorado. Una de ellas es el control de la población de su vector. La disminución de la población anofelina en las zonas de transmisión ha generado distintos resultados. El uso masivo de insecticidas químicos ha mostrado limitaciones debidas a su potencial de contaminación y a su relativa dificultad de puesta en práctica. Las medidas locales para disminuir a la población de mosquitos *Anopheles* que consisten en la eliminación de aguas estancadas (Meek S. R., 1995) y en el uso de redes impregnadas de insecticida (Hii J. L. et al., 2001), son exitosas en zonas y en las épocas de baja trasmisión de malaria. Sin embargo, estas medidas se revelan insuficientes en épocas de alta transmisión, cuando la población anofelina rebasa los niveles en los cuales la disminución del número de vector influye en la transmisión (Rubio-Palis Y., Curtis C. F., 1992). Por otra parte, el control mediante el uso de insecticidas biológicos, a través del uso de machos anofelinos estériles es difícil de aplicar, y el efecto no es duradero (Curtis C. F., Townson H., 1998) (Jayaraman K. S., 1997).

Es importante mencionar que la erradicación de un organismo de un biotopo se revela como una tarea muy árdua, ya que cada adaptación del insecto a los insecticidas produce un brote de población de insectos resistentes. Esto debido a que el nicho biológico que ocupaba resulta vacío. El hecho es que en los lugares en los cuales la malaria fue controlada, el nivel local de mosquitos anofelinos no ha disminuido, incluso ha aumentado (Rawlings P. et al. 1985).

En este sentido se ha intentado generar vectores *Anopheles* resistentes al *Plasmodium*, tratando de interrumpir esta vía de la transmisión. Las estrategias exploradas fueron diversas: se han estudiado variantes no competentes del mosquito *Anopheles* que encapsulan el *Plasmodium* por un proceso de melanización (Chun J. et al., 1995) (Cui L., Luckhart S., Rosenberg R., 2000); se estudió el efecto *in vitro* de un inhibidor de la quitinasa del parásito (*allosamidin*), lo que impide su establecimiento en los tejidos del mosquito (Shahabuddin M. et al., 1993); y se planteó una eventual transformación a nivel genómico del vector por los métodos de la ingeniería genética (Possani L.D. et al., 1998) (deLara Capurro M. et al., 2000) (James A.A. et al., 1999).

Esta última propuestas incluye la obtención de cepas transgénicas de mosquitos capases de expresar péptidos anti-*Plasmodium*. Esta vía de control resultaría interesante desde el punto de vista ecológico (no implicaría uso de insecticida) y por su efecto duradero.

Sin embargo, la generación de mosquitos transgénicos se enfrenta a varias dificultades como:

-la existencia de "subespecies" del mosquito que presentan diferencias a nivel genómico, pero que conservan la capacidad de transmisión de la malaria. En el momento de diseñar una metodología transgénica de adquisición de resistencia, será necesario identificar a un vector genético de amplio espectro de transformación que permite mutagenizar a los distintos tipos de *Anopheles* responsables de la transmisión de la malaria (Coetzee M., Craig M., le Sueur D., 2000).

-la forma de expresar al eventual péptido protector, sea de manera constitutiva ó inducida por la infección. En efecto el *Plasmodium* no activa de manera importante (si se compara con las infecciones bacterianas) al sistema inmune de las cepas permisivas del mosquito *Anopheles* (Lowenberger C. A. et al., 1999).

Los avances recientes en las investigaciones acerca de la genética del vector (*Anopheles*), de los péptidos antimicrobianos y de vectores genéticos que permiten expresar péptidos exógenos en insectos dejan vislumbrar la posibilidad de hacer realidad esta propuesta.

IV FOSFOLIPASAS TIPO A_2.

IV.1 Generalidades.

Las fosfolipasas pertenecen al grupo de las hidrolasas y son conocidas con el nombre genérico de *esterasas*; ya que actúan sobre los enlaces tipo éster de los fosfolípidos. Según la especificidad para uno u otro enlace, son definidas como A1, A2, B o C (**Figura 15**).

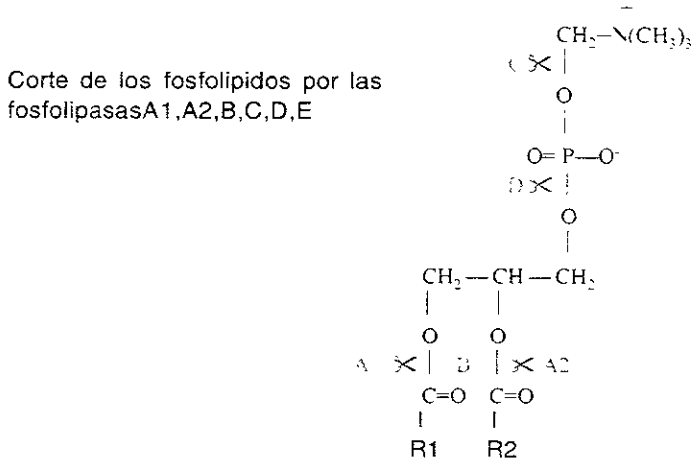

Figura 15. Esquema de los sitios de corte en los fosfolípidos por los distintos tipos de fosfolipasas.

Las fosfolipasas tipo A forman parte esencial de diversos procesos biológicos, tales como el recambio de los fosfolípidos en las biomembranas, la digestión y la producción de ácidos grasos libres (en los fagosomas), la neurotoxicidad y la citotoxicidad (en venenos de hongos, celenterados, insectos, arácnidos y reptiles), los procesos inflamatorios (por ejemplo en el ser humano), y en la reacción inmunitaria anti-microbiana (FLA2 de ratón) (Harwig S.S.L. et al., 1995).

Las fosfolipasas tipo A2 (o FLA2) definidas como E.C.3.1.1.4., catalizan la hidrólisis del enlace acil-éster situado en la posición 2 de los fosfoglicéridos, liberando ácidos grasos y lisofosfolípidos, tal y como se observa en la **Figura 15** (Van Deenen L.L.M., De Hass G.H., 1963). En las últimas dos décadas se han caracterizado a cuatro distintas formas de FLA2, las secretorias (o sFLA2), las citosólicas (o cFLA2), las fosfolipasas independientes de calcio (o iFLA2) y las acil-hidrolasas específicas del factor activador de plaquetas (o PAF) (Dennis E., 1997). Entre las fosfolipasas A2 existen numerosas familias estructurales con diferencias fisicoquímicas marcadas, como su estado de agregación (monomérica o dimérica), su especificidad por el substrato o su punto isoeléctrico (que varía de 4.0 a 11.0).

En sistemas animales, las fosfolipasas tipo A2 se activan en respuesta a hormonas, a factores de crecimiento o a moléculas agonistas que están relacionados con un gran número de tipos celulares. El ácido araquidónico es el ácido graso más comúnmente liberado por la catálisis enzimática. Esta molécula es utilizada para la síntesis de eicosanoides

(como las prostanglandinas, los troboxanos y los leucotrienos), que a su vez modulan la contracción muscular, promueven el estrés localizado y las respuestas inflamatorias (Kishino J. et al., 1994; Ohara O. et al., 1995).

Algunas FLA2 han sido documentadas por su actividad anti-microbiana. La FLA2 humana (o hsFLA2) que pertenece al grupo IIa, participa en la actividad anti-microbiana a través de dos mecanismos: por la desestabilización de la membrana a través de la penetración de la enzima en la misma; y por la destrucción de la membrana plasmática del microorganismo a través de su actividad enzimática. La especificidad de esta fosfolipasa para las membranas bacterianas se debe a su afinidad por la carga negativa de la membrana de los microorganismos, con respecto a la carga neutra de los fosfolípidos de las membranas de eucariontes. La hsFLA2, a pH fisiológico, presenta numerosos aminoácidos que están cargados positivamente, lo que pudiera explicar su especificidad por las membranas bacterianas (Buckland A.G. et al., 2000). Es notable que tras la remoción de los peptoglicanos de la membrana de bacterias Gram-negativas, se suple la actividad anti-microbiana con otros tipos de FLA2 (Qu X-D et al., 1996).

Las fosfolipasas A2 que se han encontrado en los venenos de insectos y de artrópodos comparten algunas características entre sí. Todas ellas presentan un bajo peso molecular (el cual fluctúa entre 12 a 18 kDa), contienen varios puente disulfuros (de 4 a 8), y dependen de calcio o de un catión divalente para llevar a cabo su actividad (Lambeau, G. y Lazdunski, M., 1999). Cuando se realiza un alineamiento de las secuencias peptídicas de las fosfolipasas A2, se muestra que, de manera general, tanto el sitio activo como los sitios de quelación de Ca^{2+} se conforman por los mismos aminoácidos, los cuales están ubicados en los mismos sitios relativos. La actividad citolítica de las fosfolipasas A2 del veneno del abejorro *Megabombus pennsylvnicus* se incrementa por la presencia de péptidos anfipáticos llamadas bombolitinas parecidos a las cecropinas (Peggion et al., 1998). Esta cooperatividad entre los péptidos líticos y las fosfolipasas presentes en ciertos venenos, pudiera ser cierta para los venenos de otros animales ponzoñosos.

IV.2 Clasificación de las fosfolipasas tipo A2.

Este tipo de enzimas han sido clasificadas con respecto a su patrón de cisteínas, su peso molecular, su secuencia de aminoácidos, su dependencia por el calcio y su localización dentro de la célula (Dennis, 1997).

Sin embargo, debido a la reciente proliferación de reportes acerca de las características bioquímicas y estructurales de las FLA2, se exige la ampliación de la clasificación actualmente disponible. En la **Tabla 1** se muestra la clasificación de las fosfolipasas tipo A2 descrita en 1997 por Dennis que está complementada por los nuevos grupos anexados por Lambeau y Lazdunski en 1999.

Grupo		Fuentes	Localización	Tamaño (KDa)	[Ca 2+]	nº puentes disulfuro	Amino ácidos conservados en sitio catalítico
I	A	Cobras	Secretada	13-15	mM	7	His-Asp
	B	Páncreas humano/ porcino	Secretada	13-15	mM	7	His-Asp
II	A	Víbora de cascabel, líquido sinovial, plaquetas	Secretada	13-15	mM	7	His-Asp
	B	Víbora de Gabón	Secretada	13-15	mM	6	His-Asp
	C	Ratas, testículos de ratón	Secretada	15	mM	8	His-Asp,
III	A	Abejas y lagartos	Secretadas	16	mM	5	His Asp
	B	Alacrán *Pandinus imperator*	Secretada	16	mM	5	His-Asp
IV		Hígado de ratón, plaquetas	Citosólica	85	µM		Ser 228 Arg200 y Asp549, Ser 505
V		Corazón, pulmón de humano, rata y ratón, macrófagos	Secretada	14	mM	6	His-Asp
VI		Macrófagos, células CHO	Citosólica	80-85	0	0	GlyXSerXGly
VII		Plasma de rata y ratón	Secretada	45	0	0	GlyXSerXGly, Ser 273, Asp296, His 351
VIII		Plasma de humano	Citosólica	104	0	0	
IX		Caracol marino	Secretada			0	His-Asp
X		Humano y rata	Secretada	14	mM	0	

Tabla 1. Clasificación de las fosfolipasas tipo A2 , en los amino acidos catalíticos se utiliza el codigo de tres letras , con X significando cualquier amino acido, [Ca 2+] significa: concentración de calcio requerida para la actividad fosfolipásica (*Dennis*, 1997; *Lambeau y Lazdunski*, 1999).

IV.3 Fosfolipasas tipo A2 en venenos.

Uno de los primeros trabajos sobre el análisis de fosfolipasas en venenos fue el estudio de la ß-bungarotoxina del veneno de la serpiente *Bungarus multicintus*. Esta toxina es un heterodímero que se compone por una FLA2 de 120 aminoácidos de longitud y por una subunidad denominada Kunitz, que está conformada por 61 aminoácidos. Ambas subunidades se encuentran unidas por un puente disulfuro (Kondo K. et al., 1982a). La subunidad Kunitz confiere especificidad al receptor de acetil-colina, mientras que la otra subunidad provee la actividad de fosfolipasa. Se han descubierto varias fosfolipasas diméricas, isoformas de la ß-bungarotoxina, en el veneno de esta serpiente (Kondo K. et al., 1982 b).

Además de las numerosas fosfolipasas tipo A2 que han sido extraídas de venenos de serpientes, se han purificado fosfolipasas a partir de venenos de insectos, como de la abeja (*Apis melifera*) **figura 16**; (Shipolini R.A. et al., 1976) o del abejorro (*Bombus pennsylvanicus*); (Hoffman D.R., 1994). La fosfolipasa A2 de la abeja, además de su actividad enzimática, se caracteriza por una actividad de bloqueo sobre los receptores de tipo N (Nicolas J.P. et al., 1997). Las estructuras primarias de estas fosfolipasas tienen muchos puntos en común con la fosfolipasa de alacrán mencionada en esta tesis. En el **Anexo V** se muestra el alineamiento de las secuencias aminoacídicas de algunas FLA2.

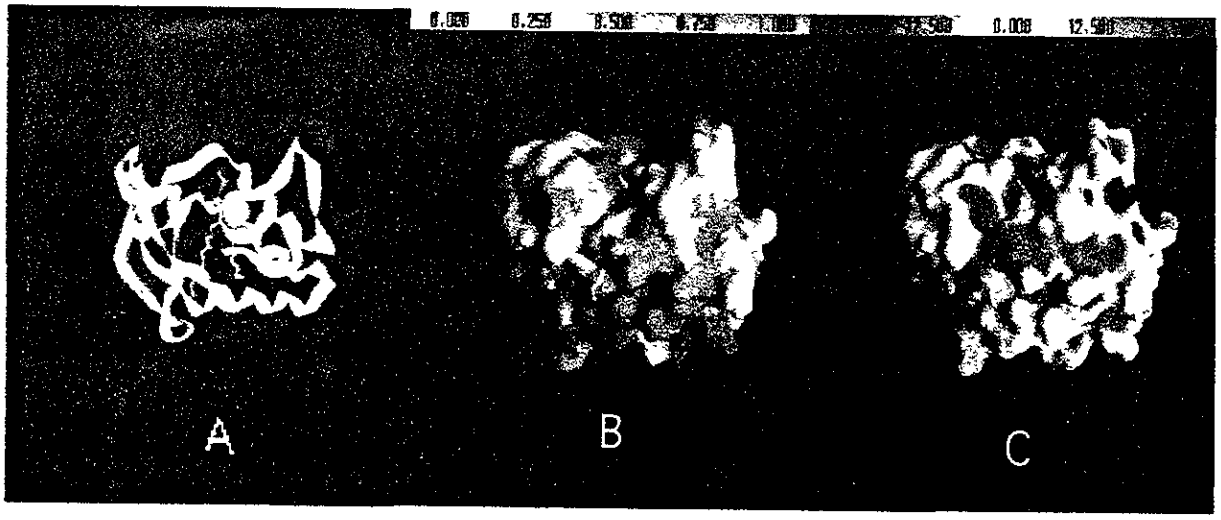

Figura 16: Representación de la estructura de la PLA2 de abeja (Scott D L et al. (1990)). A: representación con listones B: representación en función de la hidrofobicidad (O=residuo apolar, 1=residuo polar). C: representación en función del potencial de superficie (Azul: potencial negativo, rojo potencial positivo)

IV.4 Fosfolipasas tipo A2 heterodiméricas.

Hasta el momento, se han descrito sólo cinco fosfolipasas heterodiméricas. Dos de ellas purificadas a partir del veneno de serpientes, dos más purificadas a partir del veneno del alacrán *Pandinus imperator* y una purificada a partir del veneno del caracol *Conus magus*. Las dos fosfolipasas purificadas del veneno de serpiente se caracterizan por presentar una subunidad que posee funcionalidad propia Tal es el caso de la ß-bungarotoxina, en donde

su subunidad pequeña, por sí misma, es capaz de bloquear de manera específica a canales de potasio (Benishin, G., 1990). Dentro de esta clase de fosfolipasas existen dos ejemplos reportados en alacranes, que conforman la familia llamada IIIB: la Imperatoxina uno (Zamudio et al., 1997) y la fosfolipina, que forma parte de esta tesis.

La Imperatoxina I ha sido descrita por su actividad indirecta de bloqueo del canal de calcio sensible a Rianodina. Los ácidos grasos liberados por la imperatoxina I, y particularmente del ácido araquidónico bloquean el canal (Zamudio et al., 1997).

El canal de calcio sensible a rianodina es un canal intracelular del retículo sarcoplásmico de células musculares cardiacas y esquéletico. El retículo sarcoplásmico sirve como almacén de los iones Ca^{++} tomados del citoplasma y de las miofibrillas. La liberación de calcio intracelular por el canal de calcio sensible a rianodina es la mayor responsable de la contracción muscular. La depolarización de la membrana plasmática de las células musculares produce la apertura de canales de Ca^{++} en las membranas del retículo sarcoplásmico y la liberación de iones calcio del retículo sarcoplásmico hacia el citoplasma, disparando así la contracción muscular. Esta liberación de calcio ocure a través de arreglos tetraméricos de receptores de rianodina que hacen un puente entre la membrana del túbulo-T y el retículo sacoplásmico.

OBJETIVOS.

Objetivos generales.

En este trabajo se plantearon como objetivos generales,

1) El aislamiento y la caracterización de distintos componentes que presentan actividad fosfolipásica y anti-microbiana contenidos en la Fracción II de la separación con *Sephadex*^{R} del veneno del alacrán *Pandinus imperator*.

2) La clonación de las secuencias nucleotídicas que codifican para estos péptidos.

Objetivos particulares.

Aislar una fosfolipasa A2, llamada en adelante fosfolipina, así como un péptido antimicrobiano, llamado en adelante escorpina, del veneno total del alacrán *Pandinus imperator*.

Determinar la secuencia de amino-acidos de los dos componentes aislados previamente.

Analizar la actividad fosfolipásica y la actividad antimicrobiana de los componentes aislados previamente.

Analizar la capacidad de desplazamiento de rianodina en el canal de calcio sensible a rianodina del componente fosfolipásico para compararla con la capacidad de desplazamiento de rianodina de la imperatoxina I, una fosfolipasa A2 del veneno del mismo alacrán que bloquéa de manera indircta este canal (Zamudio et al. 1997).

Analizar la estructura primaria del componente fosfolipásico y compararlo con la imperatoxina I (Zamudio et al., 1995) con la finalidad de estudiar con mayor detalle las razones que provocan una especificidad distinta en el bloqueo del canal de Rianodina.

Clonar y analizar la secuencia nucleotídica de la fosfolipina.

Clonar y analizar la secuencia nucleotídica de la escorpina, con la finalidad de producirla de manera recombinante mediante el uso de vectores de expresión, y de esta forma, facilitar el análisis funcional de este péptido.

Permitir la generación de *Drosophila* recombinantes que sean capases de producir a la escorpina de manera constitutiva, con el objeto de comprobar la posible utilidad del componente anti-microbiano en la generación de mosquitos *Anopheles*

transgénicos resistentes a la infección por *Plasmodium.*

Estos objetivos fueron llevado a cabo mediante las técnicas siguientes:

La purificación de los componentes del veneno se realizó por separación sucesiva mediante filtración molecular, cromatografía de intercambio iónico y cromatografía líquida de alta presión.

La secuenciación de los péptidos se realizo en un secuenciador Milligen/Biosearch 6400/6600.

La clonación de los ADNc de la fosfolipina y de la escorpina se realizó mediante: -la generación de un banco de ADNc de télson de alacrán *Pandinus imperator* en fago lambda modificado; la búsqueda de las clonas en este banco con oligonucleótidos específicos marcados.

-RT-PCR y RACE usando un ARNm extraído de dos telsones de *Pandinus imperator.*

El análisis de la actividad fosfolipásica se hizo mediante ensayo de digestión de yema de huevo en placas de agarosa (Haberman, E. and Hardt, K. L., 1972) y digestión de substratos (fosfolípidos) marcados radioactivamente.

El análisis de la actividad antimicrobiana se realizó mediante ensayo de zonas de inhibición de crecimiento; y la actividad antiplasmódica se midió con el protocolo descrito en el articulo de Rodriguez M.C. et al. (1995). Brevemente; se cultivaron clonas de gameto de *P. berghei* cepa Anka 2.34 en ratones BALB/c por transmisión mecánica. La sangre del ratón fue colectada de 8 a 10 días después de la infección y los leucocitos fueron eliminados. Se diluyo la sangre 1 a 5 con medio de cultivo, y alicuotas de 100 µl fueron mezcladas con distintas cantidades del péptido probado. 10 y 30 minutos después, se analizó el número de rosetas formadas (indicador de fecundación activa), y 24 horas después se analizó el número de oocinetos en frotis de sangre teñido con Geimsa.

Las comparaciones de secuencias se hicieron mediante los programas de análisis FASTA, PILEUP y ClustalX utilizando secuencias del banco de datos SWISSPROT.

RESULTADOS.

Los resultados se presentan en dos artículos como sigue:

El primer artículo describe la purificación, la caracterización y la clonación de una nueva fosfolipasa de tipo A2 denominada Fosfolipina encontrada en el veneno del alacrán *Pandinus imperator*, que presenta características estructurales similares a las fosfolipasas de abeja (*Apis melifera*) y de reptil (*Heloderma horridum horridum*) .

En el segundo artículo se describe la purificación, la caracterización y la clonación de un péptido anti-microbiano que se caracteriza por presentar una estructura quimérica entre la defensina y la cecropina. Este péptido fue encontrado en el veneno del alacrán *Pandinus imperator*, y tiene efecto sobre el desarrollo de las fases oocineto y ooquiste del *Plasmodium berghei*.

Finalmente se incluye una sección de datos no publicados. En estos resultados se demuestra tanto la actividad fosfolipásica diferencial de la fosfolipina, como datos relativos la secuencia nucleotídica de la escorpina que sugieren una cierta variabilidad al nivel de los péptidos señales del péptido anti-microbiano.

I Artículos publicados.

I.1_Renaud Conde, Fernando Z. Zamudio, Baltazar Becerril, Lourival D. Possani (1999) "Phospholipin, a novel heterodimeric phospholipase A2 from *Pandinus imperator* scorpion venom". *FEBS Letters*, **460**: 447-450.

En este trabajo nos propusimos purificar y caracterizar a uno de los componentes del veneno del alacrán *Pandinus imperator* que tiene actividad fosfolipásica. Se determinó la especificidad de actividad enzimática del péptido sobre los fosfolípidos como siendo una fosfolipasa A2, así como su secuencia peptídica y su secuencia nucleotídica.

Mediante el uso de tres pasos de cromatografía (exclusión molecular, intercambio iónico y fase reversa), logramos purificar a niveles de homogeneidad a un componente de naturaleza peptídica. Usamos el ensayo de actividad fosfolipásica de descrito por Haberman E. (Haberman E., Hardt K.L., 1972) para monitorear los pasos de purificación. El péptido resultó estar constituído por dos cadenas polipeptídicas. Se secuenciaron las dos cadenas polipeptídicas mediante el uso de la técnica de degradación automatizada de Edman. La enzima es conformada por una cadena corta compuesta por 17 aminoácidos y una cadena larga conformada por 108 aminoácidos. Esta última cadena peptídica mostró similitud con la fosfolipasa A_2 obtenida a partir de veneno de abeja. La mayor homología a nivel de secuencia peptídica de este componente es con la imperatoxina I (Zamudio F. Z. et al., 1997). Este componente fue llamado fosfolipina.

Se preparó una biblioteca de cDNA a partir de las glándulas del telson del alacrán y se clonó el gen que codifica para la fosfolipina, confirmándose así la secuencia de aminoácidos de la fosfolipina que fue obtenida por la secuenciación peptídica.

Al igual que la imperatoxina I, el cDNA que codifica para la fosfolipina presenta la secuencia nucleotídica de las dos cadenas peptídicas, con un penta-péptido de procesamiento entre las dos cadenas. El extremo 5' no codificable tiene la particularidad de ser muy extenso (296 pb), si se asume como péptido señal la región que se propone en este artículo. La presencia de esta secuencia no ha sido aclarada aún.

Una copia del articulo publicado (ver las páginas siguientes) forma parte de los resultados de esta tesis.

En la sección de anexos se incluye copia de otro articulo publicado, en el cual se describió el aislamiento de la Imperatoxina I.

Zamudio, F., Conde, R., Arevalo, C., Becerril, B., Brian, M., Valdivia, H., Possani, L., (1997). The mechanism of inhibition of Ryanodine Receptor Channels by Imperatoxin I, a heterodimeric protein from the scorpion *Pandinus imperator*. *J. Biol. Chem.* **272** (18): 11886-11894.

FEBS 22828

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Phospholipin, a novel heterodimeric phospholipase A2 from *Pandinus imperator* scorpion venom

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Abstract **The primary structure of a phospholipase A2, with unique structural and functional characteristics, was determined. The large subunit has 108 amino acid residues, linked by a disulfide bridge to the small subunit, which contains 17 residues. Its gene was cloned from a cDNA library. The nucleotide sequence showed that the same RNA messenger encodes both subunits, separated only by a pentapeptide, that is processed during maturation.**

Key words: Phospholipase A2; Amino acid sequence; Nucleotide sequence; Scorpion venom; *Pandinus imperator*

1. Introduction

Phospholipase A2 (PLA2) cleaves the ester bond at position 2 of the glycerol moiety of phospholipids, and is widely distributed in mammals, reptiles and arthropods [1]. Recently, the old classification of PLA2 into three groups [2] was expanded according to some structural and functional features, such as the number of disulfide bridges and the kind of amino acids responsible for the catalytic activity, found in the newly discovered PLA2 [3]. Dennis [3] reports seven well-defined PLA2 groups (I–VII) and adds three additional ones (VIII–IX), not fully characterized. Apart from the general hydrolytic function of these enzymes, several specific biological functions have been associated with members of the PLA2 superfamily. Among these functions are anti-inflammatory action [4], myonecrotic and muscle damaging effects [5] and ion channel blocking activity [6]. β-Bungarotoxin and crotoxin are two other well known toxic venom components with PLA2 activity [7,8]. In the venom of the scorpion *Pandinus imperator* an interesting heterodimeric phospholipase (IpTxi) was recently described. This protein causes inhibition of ryanodine binding to the Ca^{2+} channels present in skeletal muscle, very likely due to an indirect effect caused by the fatty acid liberated by the PLA2 activity of IpTxi [6].

In this communication we report the isolation and characterization of a novel heterodimeric protein from the venom of the same African scorpion *P. imperator*. The large subunit shares sequence similarities with the phospholipase moiety of IpTxi [6] and with those of phospholipases from honeybee and *Heloderma* lizard, but has very little or no effect on the inhibition of ryanodine binding to Ca^{2+} channels. The small subunit is unique to this heterodimeric phospholipase. We propose to call it phospholipin, and assume that together with IpTxi it will constitute a new group of PLA2, number X, following the classification proposed by Dennis [2].

2. Materials and methods

2.1. Chemicals and purification procedure

All chemicals were analytical grade reagents, obtained from sources already described [6]. The venom of the scorpion was obtained in the laboratory by electrical stimulation, dissolved in double-distilled water, centrifuged at 15000×*g* for 15 min and the supernatant lyophilized. The soluble venom applied to a Sephadex G-50 column (0.9×190 cm) in 20 mM ammonium acetate buffer, pH 4.7, resolved at least five fractions, of which number II contains phospholipase activity; this fraction was further separated into a carboxymethylcellulose (CM-cellulose) column, as described earlier for IpTxi [6]. One of the sub-fractions from CM-cellulose (number 3) contains phospholipin, which was finally obtained in homogeneous form by high performance liquid chromatography (HPLC), using a C18 reverse-phase column (Vydac, Hisperia, CA).

2.2. Phospholipase assay and determination of specificity

The egg yolk-agarose system of Habermann and Hardt [9] was used to follow the presence of phospholipase activity during the purification procedure. The enzyme specificity was determined using radioactively labeled substrates, and thin layer chromatography, as described previously [10].

2.3. Amino acid analysis and microsequencing

Amino acid analysis was performed in samples hydrolyzed in 6 N HCl with 0.5% phenol at 110°C in evacuated, sealed tubes as described [6]. Reduced and alkylated phospholipin, in amounts of 100 μg each time, was cleaved independently by three different enzymes, and the corresponding peptides were separated by HPLC, using the conditions described in the legend for Fig. 1. Digestion with *Staphylococcus aureus* protease V8 was performed in 100 mM ammonium bicarbonate buffer, pH 7.8, for 4 h, at 40°C, whereas hydrolysis with endopeptidases AspN and ArgC was performed in the conditions described elsewhere [11]. Microsequence determination was performed on a 6400/6600 Milligen/Biosearch Prosequencer, using the peptide adsorbed protocol on CD Immobilon membranes [11].

2.4. Mass spectrometry determination

The molecular weight of pure phospholipin was determined by mass spectrometry, using a Kratos Kompact MALDI 3 v. 3.0.2 apparatus.

2.5. Cloning and sequencing

Two degenerated oligonucleotides encoding two different regions of phospholipin were synthesized, as previously described [6].

Oligo 1 (ATG TGG GAR TGY ACN AAR TGG TG-, where N is any nucleotide, R is A or G and Y is C or T-) corresponds to the DNA sequence of amino acids 2–10 of the large subunit, whereas oligo 2 (TGY GAR AAY GGN GTN GCN AC-) corresponds to the DNA sequence of amino acids 4–11 of the small subunit.

Total RNA was purified as reported [6]. Messenger RNA was purified following the instructions of the Hybond mAP protocol (messenger affinity paper, Amersham, RPN.1511). Synthesis of cDNA and the cloning of the cDNA library was performed as described [6,11]. The screening of the library was performed separately with oligonucleotides 1 and 2. The clone detected with oligonucleotide 1 was analyzed first. Inserts of cDNA from positive clones were amplified by polymerase chain reaction (PCR) using λ gt11 forward and reverse

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PII: S0014-5793(99)01392-7

primers. PCR products were subcloned into pBluescript (pKS) phagemid. Clones of interest were sequenced using the Sequenase kit v 2.0. (U.S. Biochemical Corp.). Oligos M13-20 and M13 reverse were used for sequencing [11].

3. Results and discussion

Fig. 1 shows the separation of soluble venom from *P. imperator*, by Sephadex G-50, CM-cellulose and HPLC. Fraction II (Fig. 1A) contains phospholipase activity. Sub-fraction 1 in Fig. 1B corresponds to IpTx1, capable of inhibiting ryanodine binding to skeletal muscle Ca^{2+} channels, as described by Zamudio et al [6], whereas sub-fraction 3 (Fig. 1B) also contains a protein with phospholipase activity, which can be further purified by HPLC (inset Fig. 1B). The major component from the HPLC chromatogram (shown by the asterisk in the inset of Fig. 1) was assumed to be homogeneous, based on results obtained from SDS-gel electrophoresis (data not shown). It contains phospholipase activity but very little, if any, action on the inhibition of ryanodine binding to its receptor, contrary to IpTx1 (data not shown). For this reason it was called phospholipin, without any additional reference to the ryanodine-sensitive Ca^{2+} channels. Mass spectrometry analysis showed that phospholipin was homogeneous, with a molecular mass of 14841.2. Based on this analysis, the molecule was expected to contain in the order of 125 amino acid residues. When this protein was loaded into the microsequencer, two amino acid residues of about the same amount showed in each cycle, suggesting that it consisted of a heterodimeric protein. Reduction and alkylation of phospholipin permitted separation of the two subunits. This was obtained by gel filtration on a Bio-gel P-30 column, using the same system described for IpTx1 [6]. Each one of the subunits was separately sequenced. Fig. 2A shows the results obtained. The short subunit was directly sequenced to the end, and contains only 17 amino acid residues, in which position 4 is occupied by a cysteine, responsible for the covalent attachment of the short peptide to the long one, through a disulfide bond. The larger subunit is composed of 108 amino acid residues and its complete sequence was determined after enzymatic cleavage. The sub-peptides were separated by HPLC (data not shown) and the overlapping sequences obtained are shown in Fig. 2A, underlining the overall amino acid sequence. Direct sequencing permitted the identification of the first 22 amino acid residues, whereas four sub-peptides obtained by cleaving with *S. aureus* protease V8 gave segments from amino acid residues in positions 20–37, 65–71, 73–88 and 101–108. The protease V8 cleavage was partial, since some of these fragments still contained internally situated glutamic acid residues, supposed to be cleaved by this enzyme, when the hydrolysis goes to completion. Additional positioning of residues was obtained by the use of two other proteinases. They correspond to the segment at positions 38–59 obtained by means of AspN protease hydrolysis, and sub-peptides 47–64 and 89–101, obtained by ArgC protease cleavage. It is worth mentioning that the specificity of ArgC was not 100% either; the batch of enzyme we used was able to cleave a lysine residue in position 46. The overall sequence determined by direct Edman degradation of the sub-peptides of phospholipin was additionally confirmed by the nucleotide sequence obtained from the cloned gene. The obtained cDNA nucleotide sequence of phospholipin is shown in Fig. 2B. The first gene cloned was performed using oligo 1 (see Section 2), designed to recognize the large subunit sequence, and the second was oligo 2. When the first cloned gene, based on the amino acid sequence of the large subunit (cloned with the help of oligo 1), was hybridized with oligo 2, specific for the short peptide, it was shown that both oligos were capable of recognizing the same clone. Under DNA sequencing analysis this initially misleading result was immediately understood. The gene that encodes both peptides is transcribed into the same mRNA, which encodes the large and the small subunit as well, separated by a pentapeptide Lys-Arg-Ser-Gly-Arg, which is processed during maturation, in quite the same way as initially demonstrated for the case of IpTx1, by our group [6]. The analysis and interpretation of Fig. 2B is still not complete, because there is a long 5′ non-translated segment of the cloned gene, whose function is still not certain. How-

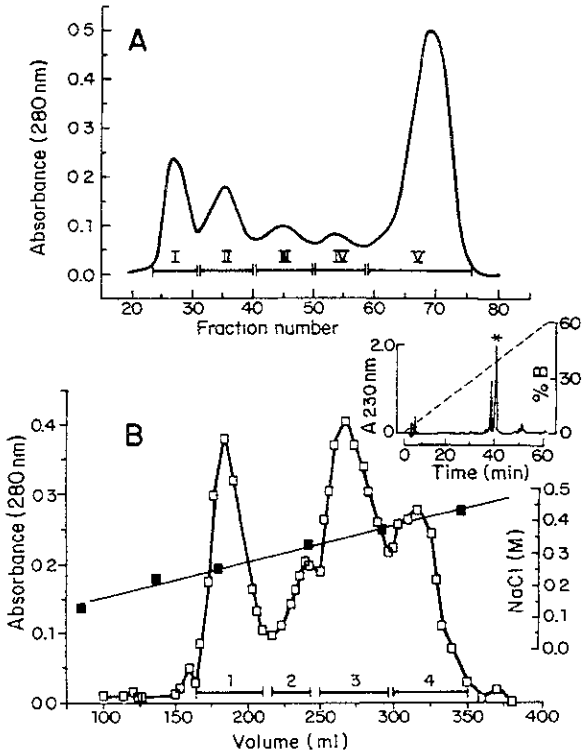
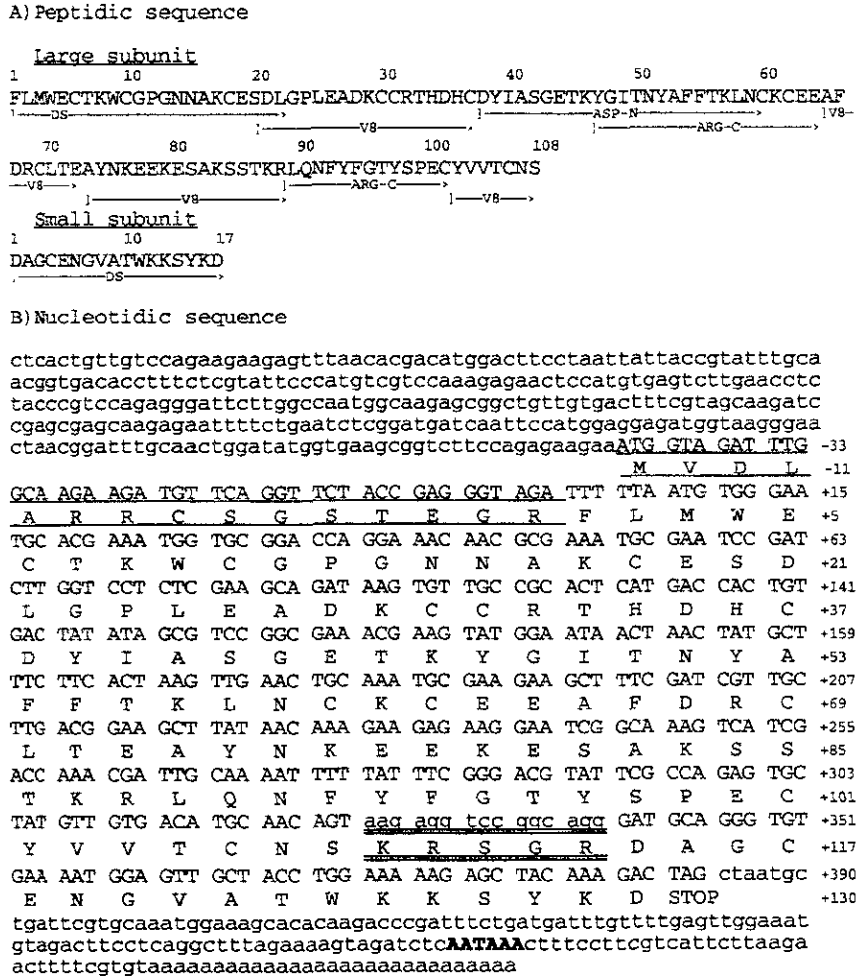


Fig. 1. Purification of phospholipin. A. Soluble venom from *P. imperator* (120 mg of protein) was applied to a Sephadex G-50 column (0.9×190 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7. Fractions of 5 ml each were collected and tested for their phospholipase activity. B. Tubes corresponding to fraction II were enzymatically active and were further applied to a CM-cellulose chromatographic column (0.9×30 cm) dissolved in the same buffer. The column was eluted with a linear gradient of sodium chloride, as indicated. Fractions 1 and 3 displayed phospholipase activity; the first contained IpTx1 [6], whereas number 3 was finally separated by HPLC in a C18 reverse-phase column, as shown in the inset. Phospholipin corresponds to the major, last sub-fraction, labeled with an asterisk. A linear gradient was used from solvent A (0.12% trifluoroacetic acid in water) to 60% B (0.10% trifluoroacetic acid in acetonitrile). Phospholipin corresponds to approximately 2.4% of the soluble venom. The experiments aimed at determining its specificity, using radiolabeled phospholipids, clearly showed that phospholipin has phospholipase A2 type activity (results not shown).

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ctcactgttgtccagaagaagagtttaacacgacatggacttcctaattattaccgtatttgca
acggtgacacctttctcgtattcccatgtcgtccaaagagaactccatgtgagtcttgaacctc
tacccgtccagagggattcttggccaatggcaagagcggctgttgtgactttcgtagcaagatc
cgagcgagcaagagaatttttctgaatctcggatgatcaattccatggaggagatggtaagggaa
ctaacggatttgcaactggatatggtgaagcggtcttccagagaagaaATG GTA GAT TTG -33
                                                  M   V   D   L  -11
GCA AGA AGA TGT TCA GGT TCT ACC GAG GGT AGA TTT TTA ATG TGG GAA +15
 A   R   R   C   S   G   S   T   E   G   R   F   L   M   W   E  +5
TGC ACG AAA TGG TGC GGA CCA GGA AAC AAC GCG AAA TGC GAA TCC GAT +63
 C   T   K   W   C   G   P   G   N   N   A   K   C   E   S   D +21
CTT GGT CCT CTC GAA GCA GAT AAG TGT TGC CGC ACT CAT GAC CAC TGT +141
 L   G   P   L   E   A   D   K   C   C   R   T   H   D   H   C +37
GAC TAT ATA GCG TCC GGC GAA ACG AAG TAT GGA ATA ACT AAC TAT GCT +159
 D   Y   I   A   S   G   E   T   K   Y   G   I   T   N   Y   A +53
TTC TTC ACT AAG TTG AAC TGC AAA TGC GAA GAA GCT TTC GAT CGT TGC +207
 F   F   T   K   L   N   C   K   C   E   E   A   F   D   R   C +69
TTG ACG GAA GCT TAT AAC AAA GAA GAG AAG GAA TCG GCA AAG TCA TCG +255
 L   T   E   A   Y   N   K   E   E   K   E   S   A   K   S   S +85
ACC AAA CGA TTG CAA AAT TTT TAT TTC GGG ACG TAT TCG CCA GAG TGC +303
 T   K   R   L   Q   N   F   Y   F   G   T   Y   S   P   E   C +101
TAT GTT GTG ACA TGC AAC AGT aag agg tcc ggc agg GAT GCA GGG TGT +351
 Y   V   V   T   C   N   S   K   R   S   G   R   D   A   G   C +117
GAA AAT GGA GTT GCT ACC TGG AAA AAG AGC TAC AAA GAC TAG ctaatgc +390
 E   N   G   V   A   T   W   K   K   S   Y   K   D  STOP       +130
tgattcgtgcaaatggaaagcacacaagacccgatttctgatgatttgttttgagttggaaat
gtagacttcctcaggctttagaaaagtagatctcAATAAActttccttcgtcattcttaaga
acttttcgtgtaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 2. Amino acid and nucleotide sequence of phospholipin. A. Primary structure of phospholipin as determined by direct sequencing of the native peptides (underlined DS) and fragments produced by enzymatic hydrolysis with protease V8 (labeled V8), endopeptidase AspN (labeled ASP-N) and endopeptidase ArgC (labeled ARG-C). B. Nucleotide sequence from the 5′ to the 3′ end of the clone that encodes phospholipin. The sequence corresponding to the putative signal is underlined, the pentapeptide eliminated during processing is double underlined, bold letters indicate the possible polyadenylation site, whereas lower case letters indicae the 5′ and 3′ non-coding regions of the gene. Numbers on the right hand side correspond to the positions of nucleotides from the signal peptide to the stop codon (upper lines) and to their corresponding amino acids (lower lines).

ever, the sequence that encodes the heterodimer phospholipin molecule is very clear. It is composed of 393 nucleotides (including the stop codon), starting at residue 1 with Phe (see line 8 in Fig. 2B) labeled on the right hand side with number +5 (which corresponds to glutamic acid), and ends at residue 130, just before the stop codon. In this sequence there is a pentapeptide (double underlined) that is processed during maturation, because it was not seen when we sequenced the heterodimeric peptides. There is a putative signal peptide (single underlined in positions −15 to 0), but there are still 304 nucleotides to the left side, with unknown function. That is, the long 5′ non-translated sequence (lower case letters) could contain information for a longer signal peptide, that might start at the ATG codon situated 9 residues to the left of the first Met residue (our amino acid −15 in Fig. 2B) implying that the signal peptide could start at amino acid −24. It is also not clear if the putative signal peptide we have labeled is, in fact, the signal peptide. We are concerned about the presence of two Arg residues within this segment. Thus, the possibility that this segment corresponds to the message for translating an unknown third peptide or long propeptide needs further analysis and work. Also, the long 3′ non-translated region (104 nucleotides), before the putative polyadenylation site (bold capital letters at the end of the figure) needs some additional work.

Although the primary structure of the large subunit of phospholipin presents a close similarity with PLA2 from honey bee and *Heloderma* lizard, which would place it in the group III phospholipases, it is not certain that it fits there. One of the main reasons is because it is a heterodimer in which the small subunit (17 amino acid residues) has no resemblance to any other protein known. It seems clear that the large subunit moiety is the one bearing the phospholipase activity, in all three examples mentioned. Also β-bungarotox-

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   1        10        20        30        40        50        60
(a)---DAGCENGVATWKKSYKD
(b)---SEECPDGVATYTGEAGYGAWAINKLNG
(c)RKRHPDCDKPPDTKICQTVVRAFYYKPSAKRCVQFRYGGCNGNGNHFKSDHLCRCECLEYR
          *  ****        *
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Fig. 3. Comparative analysis of the small phospholipase subunits. The amino acid sequence of the small subunits of phospholipin (this work), and those of IpTxi [6] and β2-bungarotoxin [13] were compared, after alignment of the first cysteinyl residues by adding gaps (-) to the sequences of phospholipin and IpTxi. Very little identity exists. Phospholipin (a) and IpTxi (b) share five identical amino acid residues at the same position (18% identity), whereas with the small β1 subunit of bungarotoxin (c) only two positions were occupied by identical residues (less than 4% identity). Bold italics indicate identical residues in all three sequences, italics indicate identity for only two of the sequences on pairwise comparison. Identical residues are highlighted by asterisks.

in, a presynaptically active phospholipase isolated from the snake *Bungarus multicinctus* [7], and IpTxi, isolated from the scorpion *P. imperator* [6] are heterodimers, but again there is no resemblance in structure or function, when we compare the small subunits of these three venom components, as shown in Fig. 3. Also functionally, the short peptide of β-bungarotoxin with 61 amino acid residues was suggested to be a K^+ channel blocker [12], whereas that of IpTxi (27 amino acid residues) apparently has no measurable function towards Ca^{2+} channels or towards any other of several ion channels assayed (see [6]). Phospholipin has the shortest of all the small subunit peptides of the three heterodimeric phospholipase known, and we do not know either what its function is. The three-dimensional structure of phospholipin was modelled using the Swissmod program and facilities (data not shown), and the results of the model suggests that cysteine at position 4 of this 17 amino acid peptide is bound to cysteine in position 101 of the phospholipase moiety. Structurally, phospholipin shows two additional cysteines (one more disulfide bridge), not present in IpTxi, which might be responsible for folding phospholipin in a different manner than that of IpTxi. It can be speculated that this structural difference is responsible for a distinct substrate specificity, hence a different biological action, such as the indirect effect on the binding of ryanodine to the Ca^{2+} channels, described for IpTxi. Due to these characteristics, phospholipin described here, although similar to group III phospholipases A2, seems to constitute the prototype of a novel group of PLA2, which would be number X, according to the classification recently proposed by Dennis [3].

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I.2 Renaud Conde, Fernando Z. Zamudio, Mario H. Rodríguez, Lourival D. Possani (2000) "Scorpine, an anti-malarial and antibacterial agent purified from scorpion venom". *FEBS Letters*, **471**: 165-168.

En este trabajo nos propusimos purificar y caracterizar a uno de los componentes del veneno del alacrán *Pandinus imperator* que tiene actividad antibiótica. Se determinó la especificidad del péptido por los microorganismos, así como su secuencia peptídica y nucleotídica.

Utilizando tres pasos de cromatografía (exclusión molecular, intercambio iónico y fase reversa), logramos purificar a homogeneidad a un componente que llamamos escorpina.

Se determinó la actividad microbicida de este péptido contra *Plasmodium berghei* con el ensayo *in vitro* descrito por Rodriguez (Rodriguez et al., 1995), y contra *Bacillus subtilis* y *Klebsiella pneumoniae* por ensayo de inhibición de crecimiento en placas de agar (Hultmark et al., 1982). Las fases oocineto y ooquiste de *Plasmodium berghei* fueron sensibles al péptido a partir de concentraciones micromolares, mientras que la actividad antibacteriana fue de aproximadamente 1μM con *Bacillus subtilis* y 10 μM con *Klebsiela pneumoniae.*

Se secuenció el péptido mediante el uso de la técnica de degradación automatizada de Edman. La secuencia peptídica de la escorpina presenta semejanza con dos clases distintas de péptidos anti-microbianos. Por una parte, la región amino terminal muestra similitud con los péptidos alfa-helicoidales anfipáticos; por otra parte, la región carboxilo terminal muestra semejanza con las defensinas de insectos.

Se preparó una biblioteca de cDNA a partir de las glándulas del alacrán y se clonó el gen que codifica para la escorpina, utilizando oligonucleótidos específicos marcados con radioactividad. Se confirmó de esta manera la secuencia de aminoácidos de la escorpina que fue obtenida por secuenciación directa. El cDNA así obtenido permitiría la expresión subsecuente de este péptido en otros organismos.

Se discute la posible utilización de la escorpina como candidato para la realización de mosquitos *Anopheles* transgénicos que resistan al parásito *Plasmodium.*

Scorpine, an anti-malaria and anti-bacterial agent purified from scorpion venom

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Abstract A novel peptide, scorpine, was isolated from the venom of the scorpion *Pandinus imperator*, with anti-bacterial activity and a potent inhibitory effect on the ookinete (ED_{50} 0.7 μM) and gamete (ED_{50} 10 μM) stages of *Plasmodium berghei* development. It has 75 amino acids, three disulfide bridges with a molecular mass of 8350 Da. Scorpine has a unique amino acid sequence, similar only to some cecropins in its N-terminal segment and to some defensins in its C-terminal region. Its gene was cloned from a cDNA library.

Key words: Antibiotic; Malaria; Scorpine; *Pandinus imperator*; *Plasmodium*

1. Introduction

Scorpion venoms are rich sources of peptides with a variety of pharmacological functions, specially those that affect membrane permeability for Na^+, K^+, Ca^{2+} and Cl^- of excitable and non-excitable cells (review [1]). They have been excellent tools to study ion-channel structure and function [2]. However, these venoms contain other interesting peptides, such as phospholipin [3] and imperatoxin-I [4], both with phospholipase activity, and the anti-microbial and anti-malaria peptide described here. Other peptides that could be effective in the control of malaria, a parasitic disease caused by *Plasmodium spp* and transmitted to vertebrates by mosquitoes, were also investigated lately [5,6]. The long range aim of the work is to incorporate into mosquitoes the genes encoding the bioactive peptides to produce transgenic vectors resistant to malaria [7]. Among the peptides isolated from the venom of the African scorpion *Pandinus imperator*, we identified a molecule, named scorpine. Due to the hybrid similarity of this peptide with the amino acid sequence of known cecropins and defensins [8], the idea came of assaying its effect on microbial growth and *Plasmodium berghei* stages that develop in mosquitoes. The description of the structure and biological function of scorpine is the object of this communication.

2. Materials and methods

2.1 Chemicals and purification procedure

All chemicals were analytical grade reagents, obtained from sources already described [3,4]. The venom of the scorpion was obtained in the laboratory by electrical stimulation, and prepared for chromatographic separations as previously reported [4]. The soluble venom applied to a Sephadex G-50 column (0.9×190 cm) in 20 mM ammonium acetate buffer, pH 4.7, resolved five well-defined fractions, from which fraction number II was subsequently applied to a carboxymethyl-cellulose (CM-cellulose) column, as described earlier for the isolation of imperatoxin-I [3] and phospholipin [4]. Scorpine was finally obtained in homogeneous form by high performance liquid chromatography (HPLC) separation, using a C18 reverse-phase column (Vydac, Hisperia, CA, USA).

2.2 Amino acid sequence

Scorpine was sequenced either as a native peptide or after reduction and alkylation, using the method described [3,4]. An aliquot (100 μg) of alkylated peptide was cleaved with endopeptidase AspN and separated by HPLC (data not shown), following the conditions described elsewhere [4]. Each independent sub-peptide was placed into the sequencer for obtaining overlapping amino acid sequences. Microsequence determination was performed on a 6400/6600 Milligen/Biosearch prosequencer, using the peptide adsorbed protocol on CD Immobilon membranes [3,4].

2.3 Mass spectrometry determination

The molecular weight of pure scorpine was determined by mass spectrometry, using a Kratos Kompact MALDI 3 V 3.0.2, apparatus.

2.4 Cloning and sequencing

Isolation of RNA and preparation of a cDNA library was performed as described [9]. The screening of the library was performed using a degenerated oligonucleotide (ATG GCN AAY ATG GAY ATG CT, where N means any nucleotide and Y means T or C), which encodes for the amino acid sequence of residues 40 to 46 (Met-Ala-Asn-Met-Asp-Met-Leu) of scorpine, synthesized as previously described [6]. The clone detected with this oligonucleotide was amplified by polymerase chain reaction (PCR) using (lambda) gt11 forward and reverse primers. PCR products were subcloned into pBluescript (pKS) phagemid and sequenced using the Sequenase® kit V. 2.0 (US Biochemical corp.). Oligo M13-20 and M13 reverse were used for sequencing [9].

2.5 Anti-bacterial assays

The classical method of Fleming was used for testing the anti-bacterial activity of scorpine. *Escherichia coli* ATCC 25922, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Klebsiella pneumoniae*, were initially assayed. Inhibition zones were recorded around wells in thin agar plates with bacteria, as described by Hultmark et al. [15].

2.6 Parasites

The gametocyte-producing clone of *P. berghei* Anka strain 2.34 (kindly provided by R.S. Sinden, Imperial College of Science Technology and Medicine, UK) was used. Parasites were maintained in BALB/c mice by mechanical passage. Mice, with parasitemias ranging between 50 and 60% and gametocytemia around 10% were bled by heart puncture using heparin (100 IU/ml blood). This usually occurred after 8–10 days post-infection.

2.7 Susceptibility of *P. berghei* sexual stages to scorpine

Ookinete cultures were carried out as described [5]. Leucocyte-de-

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PII: S0014-5793(00)01384-3

pleted infected-mouse blood was suspended 1 5 in culture medium and tested in 100 µl aliquots in flat-bottom 96-well plates Scorpine at concentrations of 0.049 to 100 µM was added to triplicate wells and the number of rosettes, indicative of active fecundation, was assessed by bright field microscopy between 10 and 30 min. later The number of ookinetes were assessed 24 h later in Giemsa-stained blood smears.

3. Results and discussion

Fig. 1 shows the profile of CM-Cellulose separation of fraction 5, from the soluble venom of *P. imperator* after gel filtration on Sephadex G-50 [4]. This chromatogram shows a better resolution of that earlier reported [4], because it was applied in a much greater amount of protein and separated with a slightly different gradient. The component eluting at about 0.35 M NaCl (labeled with an asterisk) was further applied to a C18 reverse phase column providing homogeneous scorpine (marked with an asterisk in the inset-figure). Small contaminants with weak phospholipase activity were eliminated by this HPLC step Scorpine purified by this procedure corresponds to approximately 1.4% of the total venom.

Direct automatic Edman degradation of native scorpine permitted the identification of the first 38 amino acid residues of the N-terminal segment, whereas a sample of scorpine reduced and carboxymethylated, permitted the unequivocal assignment of the first 47 amino acid residues of the primary structure, as shown in Fig 2A. Two additional sub-peptides obtained by HPLC, after digestion with AspN endopeptidase (data not shown), allowed the identification of the C-terminal part of the molecule (residues in position 44–75), as well as an extended overlapping region of scorpine (residues from positions 14 to 43). Additional segments with excellent overlapping positions were also obtained, but not included in Fig. 2A, for simplification of the picture The completion of the sequence was confirmed by the results of molecular weight determination using mass spectrometry analysis. The molecular mass of scorpine, 8350 is compatible both with the direct amino acid sequence obtained from the sequencer, and that deduced from the cloned gene Fig. 2B shows the nucleotide sequence of a gene cloned using the internal oligonucleotide for hybridization and the (lambda) gt11 forward and reverse primers for amplification A clone containing 487 nucleotides was sequenced. The internal segment contains the information (indicated by capital letters) that encodes the amino acids in position 1–75 of mature scorpine, ending in the stop codon at position 76. Two additional stretches of nucleotides were also found, one situated at the 5′ region, where a putative signal peptide containing the information for 19 amino acids is shown, and another at the 3′ untranslated region, where a putative adenylation site was identified (see lower case letters in bold, in Fig 2B).

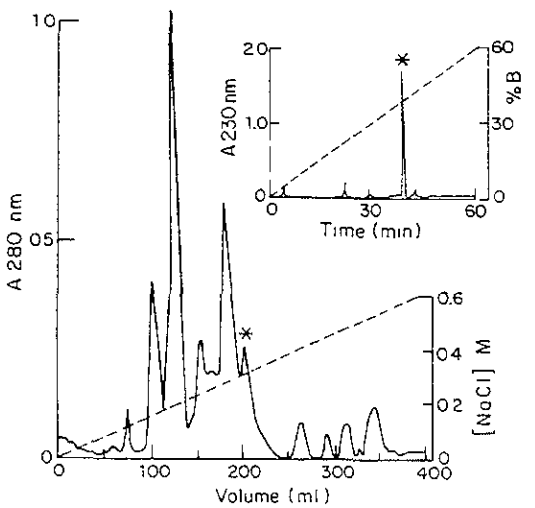

Fig 1 Purification of scorpine. Fraction II (63 mg) of the venom from *P imperator* separated by Sephadex G-50 gel filtration (see figure 1 in [4]) was loaded into a CM-cellulose chromatographic column (0.9×30 cm) dissolved in 20 mM ammonium acetate buffer, pH 4 7 The column was eluted with a linear gradient of sodium chloride, from 0.0 to 0 6 M salt The fraction labeled with an asterisk contained anti-bacterial activity, and some residual phospholipase activity and was further purified by HPLC in a C18 reverse-phase column, as shown in the inset Scorpine corresponds to the major component, labeled with an asterisk A linear gradient was used from solvent A (0 12% trifluoroacetic acid in water) to 60% B (0 10% trifluoroacetic acid in acetonitrile)

When the amino acid sequence of scorpine was compared with other known peptides, using data banks (SwissProt and FASTA program), none of them showed substantial similarities to that of scorpine, except for a small stretch of the C-terminal segment, that contains three-disulfide bridges like those of defensins [10,11]. Also, the N-terminal region was similar to cecropins [12–14]. Fig 3C shows the comparison of the primary structure of scorpine with these peptides (only representative examples were chosen for this figure). It is clear that scorpine is a unique peptide, much longer than other anti-bacterial peptides (practically double size). It seems to be a hybrid of cecropin and defensin, showing some identical amino acids (labeled with an asterisk in Fig. 2C) in certain stretches of the sequence, when appropriate gaps are manually introduced in order to increase similarities

Due to the fact that the N-terminal segment of scorpine showed similarity to cecropins, the first idea was to test its effect against bacteria.

The method described by Hultmark et al. [15] was used to access the effect of scorpine on the inhibition of bacterial growth. Clear inhibition was obtained for *B. subtilis* (Minimum inhibitory concentration (MIC) around 1.0 µM) and for *K pneumoniae* (MIC of approximately 10 µM), respectively

The biological function of scorpine in *P. imperatur* awaits investigation It is possible that it may function as microbicide within the venom gland which is open to the exterior and in contact with the hemocele of the scorpion prey during the sting.

Transmission of malaria parasites by mosquito vectors is dependent on the successful development of *Plasmodium* infective forms following ingestion of a blood-meal infected with gametocytes. This process is complex and includes a series of morphological and physiological transformations within the mosquito midgut (gametogenesis, fecundation, and ookinete formation) [16]. We previously observed in in vitro and in vivo tests that 100 µM Shiva-3, a synthetic cecropin, inhibited *P berghei* ookinetes development [5–7] The similarity of scorpine to cecropin prompted us to investigate its effect on the same *P berghei* sexual stages. Our results showed that scorpine completely inhibited both fecundation and ookinete formation at 50 and 3 µM, respectively (Fig. 3), and that it had a toxic effect on gametes and on ookinetes at

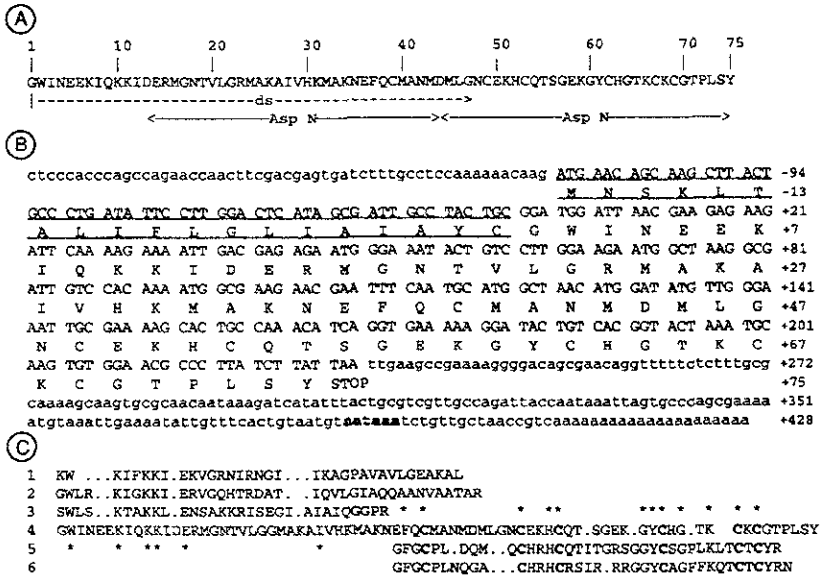

Fig 2. Amino acid and nucleotide sequence of scorpine. A: Primary structure of scorpine as determined by direct sequencing the native peptides (underlined DS) and fragments produced by enzymatic hydrolysis with endopeptidase AspN (labeled ASP-N). B: Nucleotide sequence written from the 5′ to the 3′ end of the clone that encodes scorpine. The sequence corresponding to the putative signal is underlined. The mature peptide is indicated from residues 1–75, ending with the stop codon. The lower case letter in bold at the 3′ region indicates the possible poly-adenylation site, whereas lower case letters at the extreme 5′ and 3′ ends the non-coding region of the gene. Numbers on the right side of the page correspond to the positions of nucleotides from the signal peptide to the stop codon (upper lines) and to their corresponding amino acids (lower lines). C: Amino acid sequence comparison of scorpine with that of cecropin and defensin antibiotic peptides. The sequences were aligned with extended blank spaces, because of the difference in size of the peptides and lack of extended similarities. The segments with some consensus sequences are shown pairwise with artificial blank spaces (small dots, to enhance similarity) and whenever an identical residue is situated in equivalent position, an asterisk is placed either down or up to the sequence of scorpine. The sequences compared are: cecropin B from *Antheraea pernyi* [12]; sarcotoxin Ic from *Sarcophaga peregrina* [13]; cecropin P1 from *Sus scrofa* [14], scorpine from this work, defensin from *Aeschna cyanea* [10] and a defensin from the scorpion *Leiurus quinquestriatus hebraeus* [11].

lower concentration than that of Shiva-3. The calculated ED_{50} were 10 μM for the gamete and 0.7 μM for the ookinete stages of development. These results indicate that scorpine could be a better candidate than Shiva-3, to be incorporated into the genome of genetically engineered malaria-resistant anophelines.

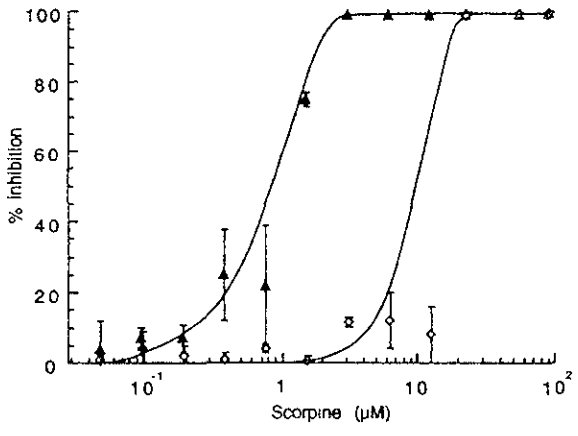

Fig 3. Dose response curve of the effect of scorpine on the ookinete and fecundation (rosette) phases of the development of *P. berghei*. Inhibition of the ookinete development (closed triangles) and inhibition of rosette formation (open rhomboids) of *P. berghei* in vitro by the scorpine peptide. For the ookinete phase, at least 10000 red blood cells were counted, whereas for the rosette formation a minimum of 10 microscopic fields were directly examined, for each scorpine concentration.

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II Datos no publicados.

II.1 Fosfolipina.

II.1a Comparación de secuencias.

Dado la naturaleza particular (hetero-dimérica) de la fosfolipina, y siendo el alacrán un animal muy antiguo, la comparación de sus fosfolipasas con las fosfolipasas encontradas en otros animales nos puede indicar que tan distinta ó semejantes son sus estructuras primarias, y eventualmente darnos algunas información sobre las posibles vías de evolución de estas enzimas. Por esta razón se realizó un alineamiento de diferentes fosfolipasas tipo A2 con las fosfolipina e imperatoxina I. En las páginas siguientes se presentan los resultados de este alineamiento. Este alineamiento se realizó con el programa FASTA (Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, Wisc.)(**Figura 17**). Se utilizaron valores de castigo para la creación de espacio de 8 y de extensión de espacio de 2. La matriz matemática usada fue la matriz *blosum50.*

Es importante resaltar que en este alineamiento se compararon las secuencias de la fosfolipina y la imperatoxina I como secuencia traducida del cDNA, sin el péptido señal, pero con el péptido unidor entre la subunidad chica y grande. Este artificio nos presenta un extremo carboxilo extendido. Se justifica incluir la subunidad chica en el alineamiento por el hecho que están codificados por un mismo ARNm y por el hecho que la remoción de la sub-unidad chica remueve la actividad enzimática (datos proporcionados por el Dr. Zamudio), lo que apunta que las dos partes de estas PLA2 son necesarias para la función.

Las secuencias peptídicas de las fosfolipasas de *Pandinus imperator*, de las fosfolipasas de *Heloderma horridum horridum* y de la fosfolipasa de abeja (*Apis melifera*), muestran más semejanzas entre sí que con las FLA2 obtenidas a partir del veneno de distintas serpientes ó con las FLA2 de mamíferos. La estructura primaria de estas fosfolipasas presentan una parte amino terminal recortada si se les compara con las fosfolipasas de serpientes y mamíferos; pero su parte carboxilo terminal es más extendida. A pesar de estas diferencias, los amino ácidos que conforman el sitio catalítico de las fosfolipasas de serpientes y de abeja se conservan en el mismo orden en las fosfolipasas de *P. imperator*. Los sitios de unión al calcio son menos conservados.

Estos datos nos permiten pensar que el mecanismo molecular de catálisis debe ser muy semejante entre estas fosfolipasas A2. La estructura primaria de la fosfolipasa de abeja es la más próxima a las fosfolipasas A2 de *P. imperator*; y se conoce su estructura tridimensional. Por otra parte, dado la gran homología entre la imperatoxina I y la fosfolipina se puede pensar que sus motivos estructurales principales serán semejantes.

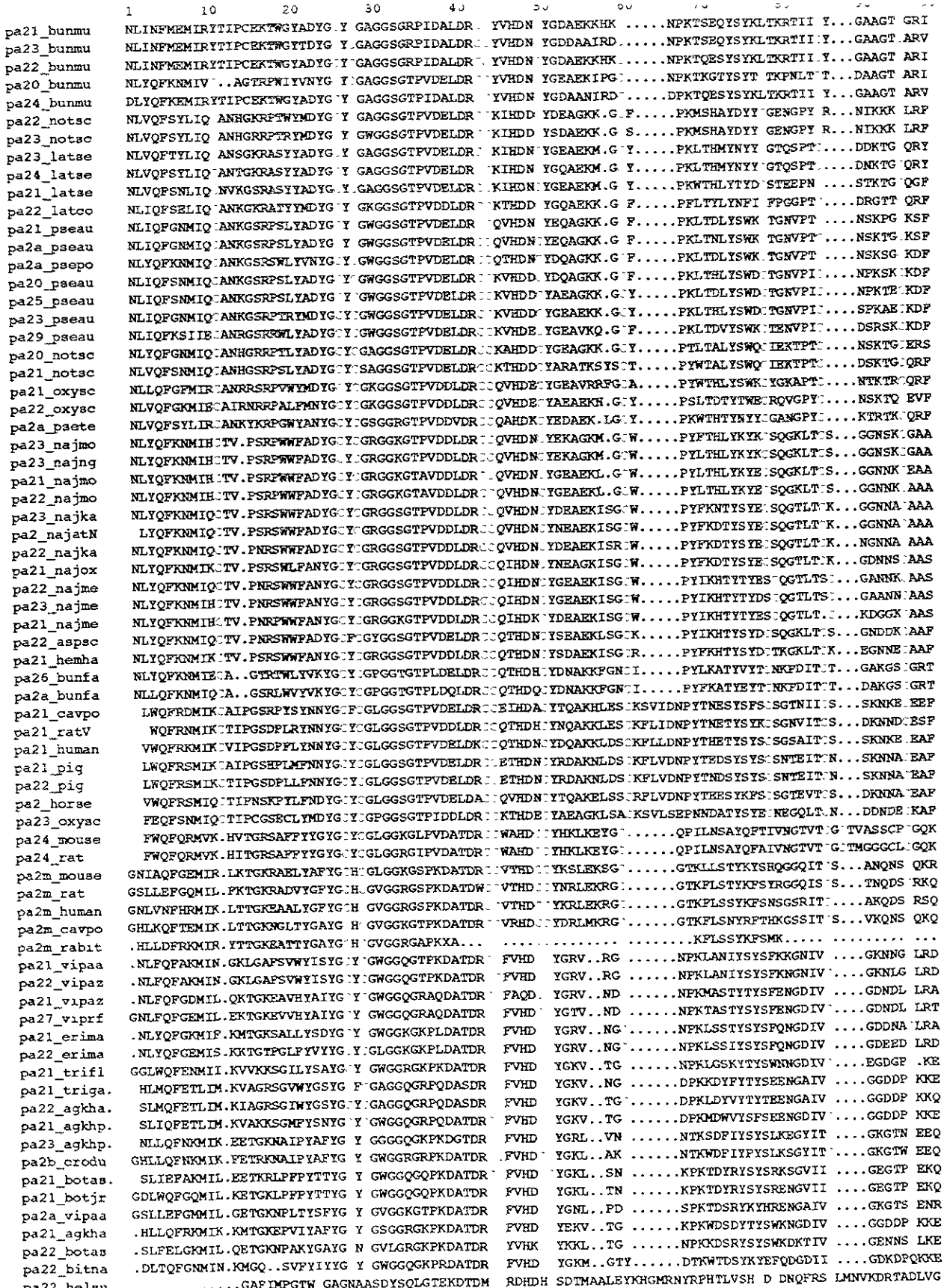
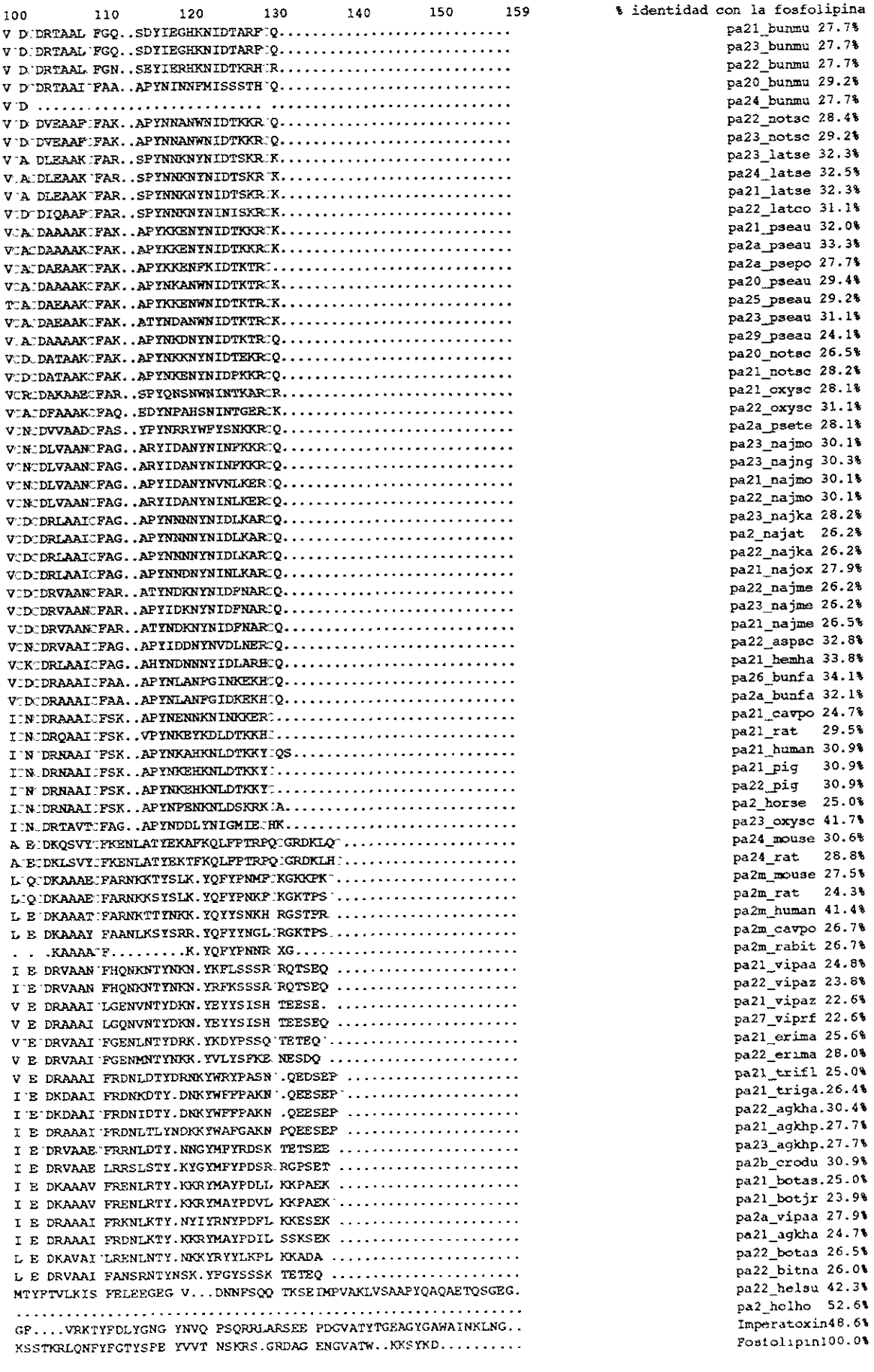


Figura 17 : Alineamento de FLA2 . Azul sitio putativo de complejamiento de calcio, Rojo : a a. involucrados en el sitio de catalisis, nombres azules FLA2 de mamifero, rojo fosfolipasas de *Pandinus imperator*



amarillo : cistelinas. Claves : azul : mamíferos, rojo : fosfolipasas de *P. imperator*.

A partir de este alineamiento de secuencias, se generó un árbol filogenético sin raíz. Para ello, se utilizó el programa ClustalX (Thomson et al.(1997)). Los resultados obtenidos se muestran en **la Figura 18**. Este árbol nos indica las relaciones estructurales; más que distancias evolutivas.

Un punto relevante de este árbol es la aparición de un grupo estructural de fosfolipasas, que integra las fosfolipasas A2 de los helodermas, asi como las fosfolipasas A2 de los *Hymenoptera*. En el anexo V se presentan diferentes fosfolipasas que pertenecen a este grupo de PLA2. Se pueden observar las distancias estructurales entre estas fosfolipasas A2 con las fosfolipasas extraidas de mamíferos y de veneno de diversas serpientes las cuales forman dos grupos estructurales de FLA2 distintos.

Sin embargo, como se pudo apreciar en el alineamiento, las diferencias no son tan grandes, a pesar de la lejania evolutiva entre serpientes, alacranes, lagartos y mamíferos.

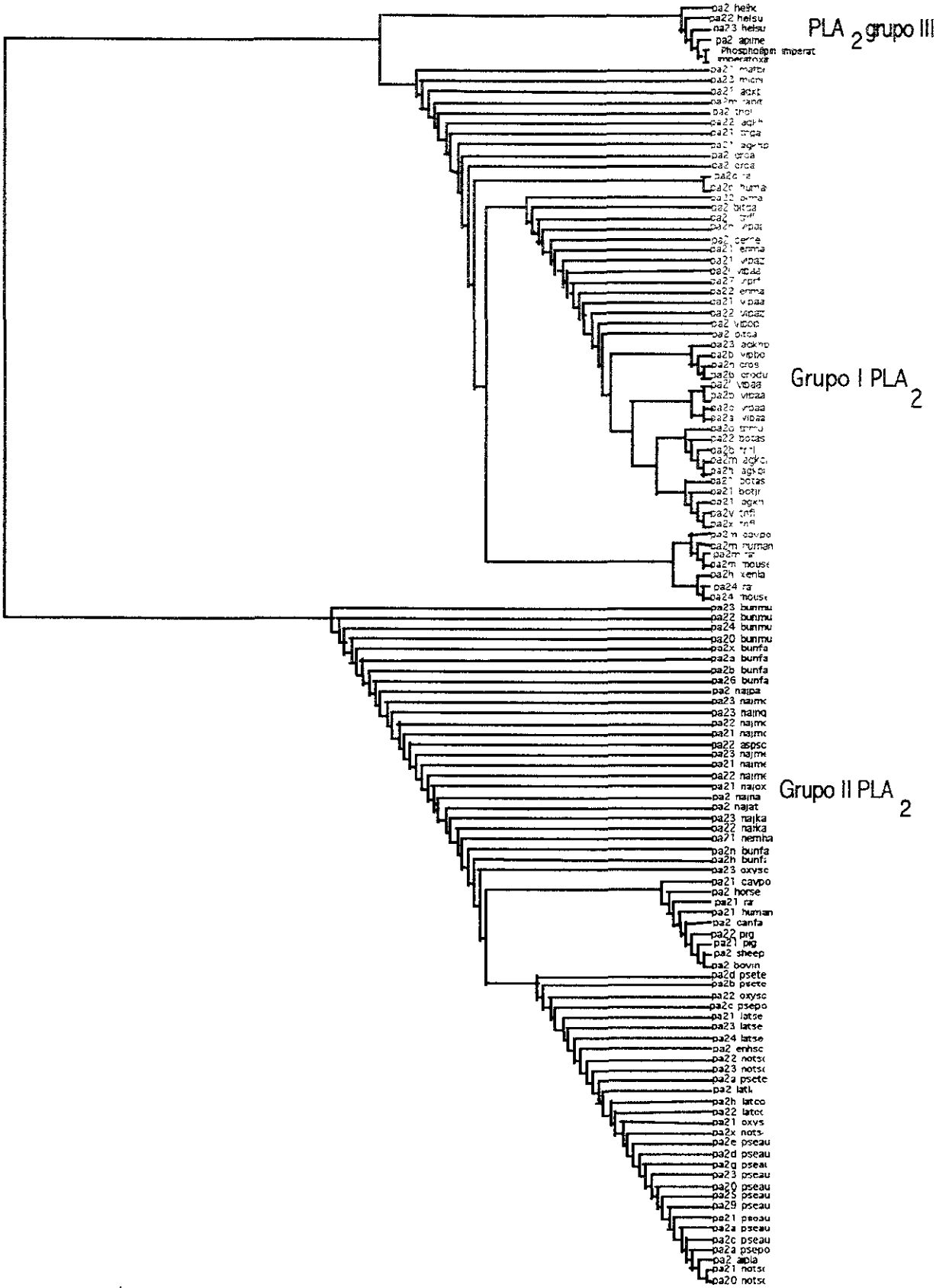
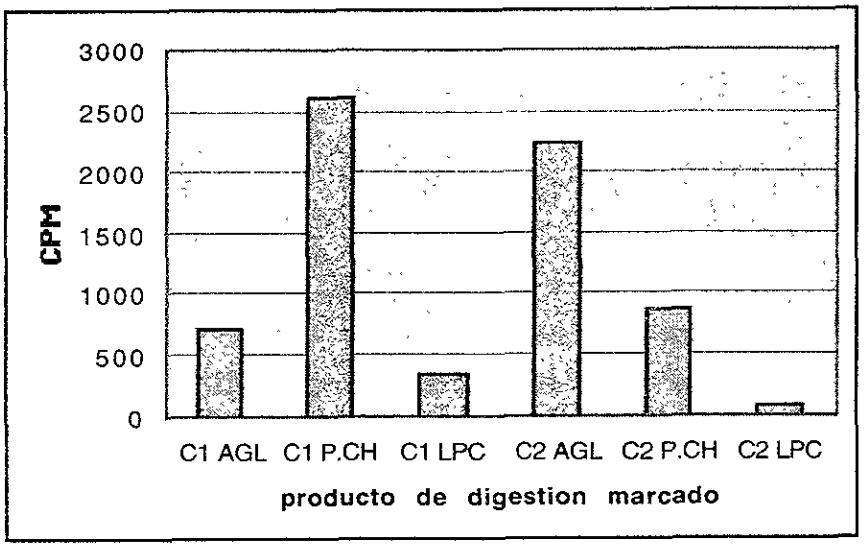


Figura 18. Árbol filogenético sin raíz realizado en base al alineamiento de las secuencias peptídicas de algunas fosfolipasas A2 de la figura 11. En el grupoIII estan las fosfolipasas A2 semejantes a la fosfolipasa A2 de abeja

II.1b Datos de actividad fosfolipásica.

Ensayo de especificida de actividad fosfolipásica.

El ensayo de especificidad de se hizo con el protocolo descrito por Sosa B.P. y colaboradores (Sosa B.P. et al., 1986). Brevemente se digerió fosfatidilcolinas marcadas radioactivamente en el ácido graso 1 ó 2; subsecuentemente se separaron los productos de las digestiones por cromatografia en capa fina, y se analizaron las zonas correspondiente a cada uno de los productos de digestión. Cuando se utilizó fosfatidilcolina marcada en el ácido graso 2, la señal radioactiva se encontró principalmente en la fracción del ácido graso libre 2 y cuando se utilizó el fosfolípido marcado en el ácido graso 1 la señal radioactiva se encontró en el lisofosfolípido y la fosfatidilcolina. Esto, demuestra que la actividad fosfolipásica de la fosfolipina es de tipo A2.



Especificidad de la Fosfolipina: C1::fosfatidilcolina marcada en el primero ácido graso, C2: fosfatidilcolina marcada en el segundo ácido graso, AGL: ácido graso libre, LPC: lisofosfatidilcolina,P. CH:fosfatidilcolina.

Ensayo de actividad fosfolipásica.

El ensayo de digestión de yema de huevo en placas de agarosa (Haberman E., Hardt K. L., 1972) fue utilizado para la detección de la actividad fosfolipásica. Brevemente, el ensayo consiste en el llenado de una caja de Petri con una capa de 2 mm de espesor compuesta por una mezcla de agarosa al 0.06% en 1.08 mM de NaCl , 1mM de $CaCl_2$, 50 mM de Tris.HCl pH 7.95 con yema de huevo al 0.245%. Posteriormente se prepara un pozo de 2 mm de diámetro que se cubre con la fracción a probar, y se incuba a temperatura ambiente durante 70 minutos. La actividad se traduce por la aparición de un halo transparente alrededor del pozo.

Al colocar 3 µg de imperatoxina I y de fosfolipina en los pozos del ensayo con yema de huevo se obtuvo un halo transparente de 5 mm y 9 mm de diámetro respectivamente. Este dato nos indica que, en las condiciones del ensayo, la fosfolipina mostró una actividad relativamente mayor a la obtenida con la imperatoxina I.

Ensayo de desplazamiento de rianodina marcada radiactivamente.

Al cuantificar la unión de la rianodina a membranas del retículo sarcoplásmico tanto de músculo cardíaco como esquelético de cerdo o de conejo; el efecto de inhibición al pegado del alcaloide fue menor en dos órdenes de magnitud con respecto al efecto observado en presencia de la imperatoxina I (datos proporcionados por el Dr. Fernando Zamudio Zuñiga).

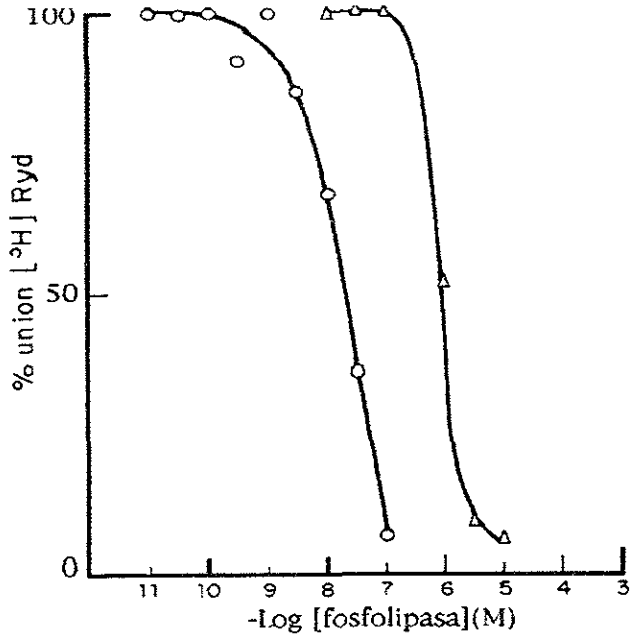


Figura 19. Desplazamiento del pegado de la rianodina en función de la concentración de la fosfolipasa probada. Círculos, imperatoxina I; triángulos, fosfolipina.

La actividad de la imperatoxina I sobre el canal de calcio sensible a rianodina ha sido descrita como una consecuencia de su actividad enzimática. El desplazamiento de la rianodina se debe a la liberación de ácidos grasos libres, principalmente del ácido araquidónico (Zamudio et al., 1997). Por lo tanto, la diferencia de la actividad sobre el pegado de Rianodina entre la fosfolipina y la imperatoxina I se debe, probablemente, a la diferencia de la actividad fosfolipásica en función del tipo del substrato utilizado.

II.1.c Conclusiones

En conclusión, los datos de análisis de estructura primaria nos indican que la fosfolipina, a pesar de tener rasgos muy particulares (la presencia de una subunidad chica ligada covalentemente a la parte grande de la fosfolipasa o la presencia de un puente disulfuro adicional si se le compara con la imperatoxina I), tiene muchos amino ácidos conservados cuando se le alinea con las demás PLA2 solubles. Las posiciones relativas de los aminoácidos que constituyen los diferentes motivos estructurales conocidos en las PLA2 son igualmente conservados en la fosfolipina. Se puede esperar que motivos de estructura secundaria sean igualmente conservados y que el mecanismo catalítico sea el mismo que en las fosfolipasas descritas anteriormente.

Las fosfolipasas A2 estructuralmente más cercanas a la fosfolipina son las fosfolipasas A2 extraídas de abeja y abejorro, y desde luego la Imperatoxina I extraída del veneno del mismo alacrán. A pesar de esto, la fosfolipina y la Imperatoxina I presentan actividad enzimática distinta que aparentemente podría deberse a diferencias de afinidad para el substrato. Estas diferencias de actividad traducen el hecho que pequeños cambios estructurales pueden afectar la especificidad de estas enzimas.

La evolución de los componentes del veneno del alacrán *P. imperator* contiene todo un arsenal protéico que, a pesar de ser estructuralmente muy relacionados, presenta actividad y posiblemente funciones distintas. Hemos encontrado en el dos fosfolipasas A2 que presentan nuevas estructuras primarias y que, a pesar de ser relacionadas estructuralmente entre ellas, presentan actividades distintas, así como características diferentes al nivel de sus ADNc.

II.2 Escorpina.

II.2.a Variabilidad de la secuencia nucleotídica.

Durante la búsqueda de la secuencia nucleotídica de la escorpina que fue realizada en el banco de fagos (Condé et al., 1999), se identificó a una secuencia nucleotídica de la escorpina que representa a un extremo 5' no codificante de longitud mayor a la secuencia publicada. Esta secuencia se presenta en la **Figura 20**.

```
5'-gaaccaacttcgacagtgaaaccctcccacccagagccagaaccaacttcgacgagt

gatctttgcctccaaaaaacaag ATG AAC AGC AAG CTT ACT GCC CTG ATA
                          M   N   S   K   L   T   A   L   I
TTC CTT GGA CTC ATA GCG ATT GCC TAC TGC GGA TGG ATT AAC GAA
 F   L   G   L   I   A   I   A   Y   C   G   W   I   N   E
GAG AAG ATT CAA AAG AAA ATT GAC GAG AGA ATG GGA AAT ACT GTC
 E   K   I   Q   K   K   I   D   E   R   M   G   N   T   V
CTT GGA GGA ATG GCT AAG GCG ATC GTC CAC AAA ATG GCG AAG AAC
 L   G   G   M   A   K   A   I   V   H   K   M   A   K   N
GAA TTT CAA TGC ATG GCT AAC ATG GAT ATG TTG GGA AAT TGC GAA
 E   F   Q   C   M   A   N   M   D   M   L   G   N   C   E
AAG CAC TGC CAA ACA TCA GGT GAA AAA GGA TAC TGT CAC GGT ACT
 K   H   C   Q   T   S   G   E   K   G   Y   C   H   G   T
AAA TGC AAG TGT GGA ACG CCC TTA TCC TAT TAA TTGAAGCCGAAAAGG
 K   C   K   C   G   T   P   L   S   Y  STOP
GGACAGCGAACAGGTTTTTCTCTTTGCGCAAAAGCAGTGCGCAACAATAAAGATCATAT

TTACTGCGTCGTTGCCAGATTACCAATAAATTAGTGCCCAGCGAAAAATGTAAATTGAA

AATATTGTTTCACTGTGAATGTAATAAATCTGTTGCTAACCGTCAAAAAAAAAAAAAAA

AAAAAAAAAAAAAAAA-3'
```

Figura 20. Secuencia nucleotídica obtenida a partir del banco de fagos recombinantes. La secuencia nucleotídica subrayada codifica para el posible péptido señal. En letras itálicas se representa a la secuencia nucleotídica del extremo 3' no codificante; la posible señal de poli-adenilación se marcó con letras negritas. y en minúculas se presenta el extremo 5' no codificante de la secuencia nucleotídica .

Ensayo tipo RACE; Obtención del extremo 5 ' de la secuencia nucleotídica de la escorpina.

Con el objeto de determinar si las variaciones de la secuencia 5' no codificantes que habían sido observadas representan un factor común de la secuencia nucleotídica de la escorpina, se implementó una búsqueda por la técnica RACE (Rapid Amplification of cDNA Ends) (Frohman M.A. et al., 1988) del extremo 5'. Para ello, se extrajo el ARN de 2 telsones del alacrán *P. imperator* mediante el uso de una metodología modificada, la cual consiste de la eliminación mecánica del caparazón de quitina de los telsones. El ARN total se obtuvo por el protocolo de precipitación con cloruro de guanidina (Strohman et al., 1977). Posteriormente, el ARNm se purificó mediante el uso de una columna de celulosa dT, de acuerdo a lo indicado por A. van Leder (Sambrook, Fritsch y Maniatis, pp 7.26-7.37) y se trató con la enzima transcriptasa reversa (*Expand Reverse Transcriptase*^{R}, Bhoeringer Manheim) usando para ello

un oligonucleótido de 15 Ts como cebador de la reacción. Con esta reacción se obtuvo la primera cadena de cDNA. Finalmente, se agregaron guanidinas a través de una reacción de transferasa terminal con el estuche comercializado por Bhoeringer Manheim^R siguiendo la metodología indicada por el fabricante.

Se utilizaron distintos oligonucleótidos con el fin de lograr una amplificación específica, en particular a los oligonucleótidos R1, R2 y Cecrop, y usando al oligonucleótido C15 como cebador en el extremo 5' de la secuencia. En la **Figura 21** se presentan tanto a las secuencias nucleotídicas de los oligonucleótidos usados como a las posiciones de cada uno de ellos dentro de la secuencia nucleotídica de la escorpina.

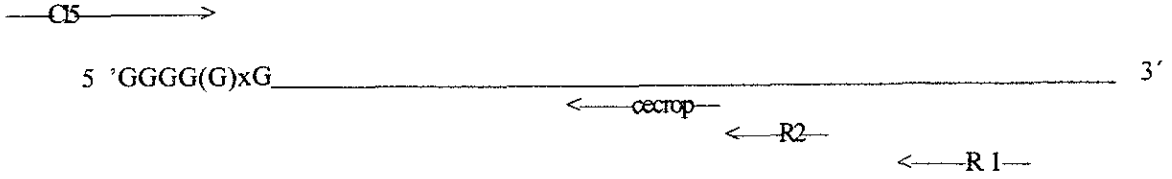


Figura 21. Esquema de las posiciones relativas de los oligonucleótidos usados en el experimento de RACE en la secuencia nucleotídica de la escorpina. Cecrop, oligonucleótido [TTATTGAAATTCGTTCTTCG] cuya secuencia nucleotídica corresponde a la secuencia nucleotídica conocida del extremo 3', 214 pb hacia el extremo 5'; R1, oligonucleótido [GCAACAGATTTATTACATTCACA] cuya secuencia nucleotídica corresponde a la secuencia nucleotídica conocida del extremo 3', 12 pb hacia el extremo 5'; R2, oligonucleótido [TACATTTTTCGCTGGGCACTA] cuya secuencia nucleotídica corresponde a la secuencia nucleotídica conocida del extremo 3', 61 pb hacia el extremo 5', todos en dirección del extremo 5' de la secuencia nucleotídica de la escorpina.

La secuencia de amplificación fue la siguiente: 35 ciclos de amplificación por PCR sobre el ADN preparado como se mencionó anteriormente, utilizando a los oligonucleótidos C15 y R1 a una temperatura de alineamiento de 54 °C. Posteriormente se eliminó a los oligonucleótidos sobrantes precipitando el ADN con acetato de amonio y con etanol. Sobre este producto se llevó a cabo una amplificación de 30 ciclos con los oligonucleótidos C15 y R2, a una temperatura de alineamiento de 54 °C. Se precipitó el ADN como se indicó previamente y se repitió la amplificación por PCR, esta vez, durante 30 ciclos con los oligonucleótidos C15 y Cecrop, a una temperatura de alineamiento de 52 °C.

Los ADNs resultantes de las amplificaciones finales fueron purificados en geles de agarosa, utilizando para ello al estuche de extracción de ADN *GlassMax*^{R} comercializado por Gibco BRL, y se usaron nuevamente para la amplificación de reto: con un único oligonucleótido y con los dos (cecrop y C15). Un fragmento de aproximadamente 250 pares de bases fue amplificado sólo en presencia de los dos oligonucleótidos, a una temperatura de 52 °C.

Este fragmento de DNA fue subclonado en el vector pKS y se secuenció. La secuencia obtenida está representada en la **Figura 22**.

```
Race  CCCCCCCCCCCCCCCAAAAACAAG ATG AAC AGC AAG CTT ACT ACT GCC CTG ATA TTC
                                 M   N   S   K   L   T   T   A   L   I   F

Race  CTT GGA CTC ATA GCG ATT GCC TAC TGC GGA TGG ATT AAC GAA GAG AAG ATT
      L   G   L   I   A   I   A   Y   C   G   W   I   N   E   E   K   I

Race  CAA AAG AAA ATT GAC GAG AGA ATG GGA AAT ACT GTC CTT GGA GGA ATG GCT
      Q   K   K   I   D   E   R   M   G   N   T   V   L   G   G   M   A

                                  <GC TTC TTG CTT AAA GTT ATT
Race  AAG GCG ATT GTC CAC AAA ATG GCG AAG AAC GAA TTT CAA
      K   A   I   V   H   K   M   A   K   N   E   F   Q
```

Figura 22. Ensayo RACE. Secuencia nucleotídica del producto de RACE. El posible péptido señal se encuentra subrayado; en letras itálicas se indica la posición del oligonucleótido cecrop.

De acuerdo con los datos obtenidos, la secuencia nucleotídica resulta casi idéntica a la secuencia nucleotídica obtenida anteriormente, salvo por el hecho de que esta última es más corta en su extremo 5', lo que puede deberse a distintas razones. La primera de ellas es que la polimerasa reversa se haya desprendido de su templado antes del finalizar la secuencia y, de esta manera, los productos más cortos se amplificarían preferencialmente. La segunda posible razón es que se trate de una secuencia distinta (quizá una isoforma) o que exista cierta variación entre los individuos analizados. Esta última propuesta pudiera sustentarse por la aparición de un residuo de treonina en el péptido señal. Es posible que exista una cierta variación de la secuencia nucleotídica de la escorpina a nivel genómico entre los individuos del alacrán *P. imperator*. Otra posibilidad que debemos considerar es que existan varias réplicas de la secuencia nucleotídica de la escorpina en el genoma del alacrán.

II.2.b Predicción de la estructura secundaria de la Escorpina.

El conocimiento de la secuencia peptídica de la escorpina no nos indica cuales son los amino-ácidos importantes para su función. Los datos relativos a la estructura secundaria de otros péptidos de secuencia peptídica semejante a la escorpina de los cuales se conocen la estructura, permiten realizar una predicción de la estructura secundaria de la escorpina y así obtener algunas pistas sobre la importancia relativa de sus amino ácidos para llevar a cabo su función. Con este fin, se utilizó un programa informático de predicción de estructura.

A continuación se presenta un análisis de la posible formación de una hélice alfa en la región amino terminal de la escorpina. La predicción de la estructura secundaria es el resultado del análisis de la secuencia primaria con el programa PHD Versión: 1.96: (Rost B, et al., 1996)(**Figura 23**).

```
AA GWIN EE KIQ KK IDE RMGNTVLGGMA KAIVHKMA K NEFQCMANM D MLGNC E KHCQTSG E KGYCHGT K CK CGTPLSY
1      HHHHHHHHHHH      HHHHHHHHHHHHHHHHHHHHHHHHHHHHH    HHHHH
2  9977588899997623741359999999999963267553323344156698671789675699985437555 69
3  LLLL HHHHHHHHHH ..L... HHHHHHHHHHHHH ..HHHH ........ LLHHHHH .LLLLLLLLLLL..LLLLLL
4  e beeeebeeebeeebeebbbbbbbebbbbebbeeebebbbbbebbeebeeebeebbeebebeeeebebbbe be
5  5101322325222312001651036278912334217082715153116241523001 46ebeeeebebbbe be
6  e........e.........bb...b.bbb....e..b.b.b.b.b...b.e.b.....eb......b.b......
```

Figura 23. Predicción de la estructura secundaria de la escorpina con el programa *Profile Network Prediction* de Heidelber, Alemania. AA, secuencia peptídica de la escorpina; 1, primera predicción (H: hélice alfa); 2, índice de probabilidad de la primera predicción; 3, predicción de la estructura con los índices de 2 >8.5 (H: hélice alfa, L: asa); 4, acceso al solvente. (b: de 0 a 9 %, i: de 10 a 36%, e: de 37 a 100%); 5, índice de seguridad del cálculo de acceso al solvente; 6, definición de acceso al solvente con una correlación superior a 0.69 (b: aminoácido embebido en la proteína , e: aminoácido expuesto en la superficie de la proteína).

En esta predicción se evidencia la posible presencia en la parte amino terminal de la escorpina de una helice α con un doblez en el residuo de glicina que se encuentra en la posición 17, lo que dividiría la región amino terminal de la escorpina en 2 zonas de hélice alfa. Cuando se atribuye a la parte amino terminal de la escorpina una estructura de helice alfa, se aprecian dos posibles vectores de anfipatía que son representados en la **Figura 24a**.

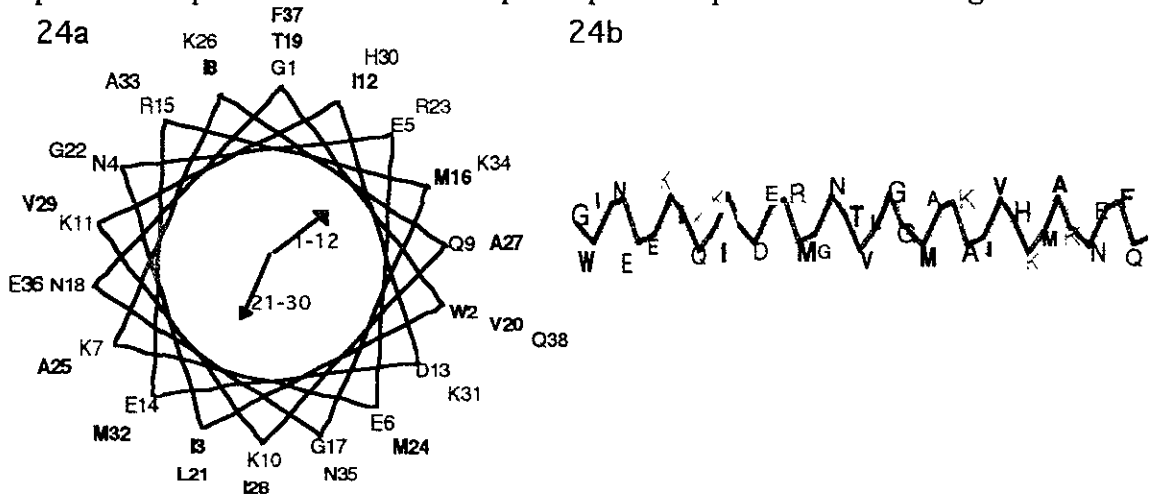


Figura 24. Representación de la distribución teórica de aminoácidos en la región amino terminal de la escorpina, asumiendo una conformación de hélice alfa perfecta en la cadena peptídica. 24a. Vista desde el eje de la hélice. 24b. Vista lateral de la hélice. En letras negritas se indican a los aminoácidos hidrofóbicos; en rojo a los aminoácidos cargados negativamente a un pH 7.0; en azul a los aminoácidos cargados positivamente a pH.7.0.

II.2.c Conclusiones

En conclusión, hemos aislado del veneno del alacrán *P. imperator* un péptido que tiene efecto antiplasmodico y antimicrobiano. Es codificado por un ARNm que varía en el extremo 5' de su secuencia nucleotídica. Su secuencia peptídica es híbrida entre dos clases estructurales de péptidos antimicrobianos : su parte amino terminal tiene semejanza con péptidos de tipo cecropina y un extremo carboxilo semejanza con péptidos de tipo defensina. En su extremo amino terminal la escorpina presenta dos vectores de anfipatía.

DISCUSIÓN.

I General.

En el veneno de los animales ponzoñosos se encuentran distintos tipos de péptidos. La presencia de péptidos líticos y de fosfolipasas constituye una combinación tóxica presente en el veneno de las abejas (melitina y la fosfolipasa A_2 de abeja (PLA2 APIME)) y de abejorros (bombolitina) que aumenta su eficacia para destruir a los tejidos de las presas. Los péptidos líticos facilitan el acceso de las fosfolipasas a su blanco, lo que podría explicar la presencia de ambas proteínas en el veneno (Segrest J. P. et al., 1990).

El veneno del alacrán *Pandinus imperator* presenta fosfolipasas con una estructura primaria particular, lo que podría traducirse en la necesidad de una enzima que destruya a los tejidos de los organismos blancos. La presencia en el veneno de un péptido de tipo anti-microbiano pudiera ser a primera vista extraña, ya que la picadura tiene como objetivo principal la muerte de las presas. La glándula ponzoñosa del alacrán tiene una apertura al medio exterior; y una infección de esta glándula resultaría sumamente peligrosa para la supervivencia del alacrán. Sin embargo, la proximidad estructural entre las toxinas del alacrán que bloquean canales de potasio y las defensinas pudiera dar una razón evolutiva de la presencia de este péptido en el veneno. Además los péptidos líticos (como la bombolitina o la melitina) presentan puntos en común con los péptidos anti-microbianos lineales de carácter anfipático (como las cecropinas); y la escorpina comparte algunas de estas características. La combinación de péptidos líticos y de fosfolipasas tipo A2 pudiera tener un efecto tóxico sinérgico, si se comparara con el efecto de cada uno de los péptidos de manera independiente.

II Fosfolipina.

La fosfolipina presenta características estructurales únicas. La analogía más cercana en cuanto a su secuencia peptídica se encuentra en la imperatoxina I, que proviene del veneno de la misma especie de alacrán. Sin embargo, sus características estructurales difieren de la Imperatoxina I, como su número de puentes disulfuro (6 en lugar de 5 en la Imperatoxina I), o su pobre efecto indirecto de bloqueo del canal de calcio sensible a la Rianodina. Estas diferencias estructurales pudieran traducirse en diferencias de afinidad por los fosfolípidos de membrana, lo que explicaría la diferencia de bloqueo.

Desde un punto de vista genético, los cDNA clonados presentan un extremo 5` no codificante muy largo (241 bases) si se compara con el 5' no codificante de la FLA2 de abeja (76 bases)(Kuchler K. et al.1989) o del mismo alacrán (10 bases) (Zamudio et al., 1997) . Esto podría traducirse en un procesamiento de varios péptidos después de la traducción de un solo ARNm, o en la ineficiencia de la secuencia nucleotídica de anclaje al ribosoma.

El modo de acción microbicida de la escorpina aún no ha sido del todo aclarado; sin embargo, a la fecha no se ha detectado un efecto tóxico sobre insectos o mamíferos. La escorpina no es letal para las moscas *Drosophila* hasta concentraciones de 0.625 μM (si se considera que todo el insecto está prácticamente constituído por agua); tampoco se ha detectado efecto alguno en grillos, acociles, o ratones (datos no publicados). Aún así, no se puede descartar que exista un canal iónico sobre el cual actúe la escorpina.

El mecanismo de la actividad plasmódica de la escorpina contra las fases oocineto y ooquiste del *Plasmodium* no han sido delucidadas aún. Su actividad sobre la fase ooquinete del *Plasmodium* se da en concentraciones del orden micromolar. Este rango de M.I.C. es semejante al rango de M.I.C. sobre bacterias de otros péptidos antimicrobianos. Se puede pensar que la escorpina actúa de la misma manera que la mayoría de los péptidos que le son estructuralmente relacionados, es decir, permeando la membrana celular del microrganismo.

La cercanía estructural que existe entre las toxinas del alacrán que actúan sobre canales de potasio y las defensinas de insectos, nos induce a tomar con mucha cautela al mecanismo de actividad anti-microbiana de la escorpina. Pudiera tratarse de una toxina que haya mutado o evolucionado hasta llegar a tener una actividad anti-microbiana, y haya perdido su actividad de pegado al receptor original. De hecho se ha descrito el proceso inverso para la defensina A, que bloquea al canal potasio dependiente de voltaje (Shimoda M. et. al., 1994).

El proceso de evolución de la escorpina podría haber sido el resultado de una duplicación del péptido señal, lo cual explicaría la presencia del extremo amino terminal de la escorpina. Otra posible explicación para este péptido con dos regiones distintas, sería el hecho de que se tratase de un péptido precursor, el cual se encontraría también en los hemocitos de la hemolinfa. El péptido precursor pudiera entonces someterse a un procesamiento previo a su liberación en la hemolinfa, que lo activaria. Este tipo de procesamiento es muy común en péptidos anti-microbianos de mamíferos (Storici P. et al., 1996).

La escorpina se detectó en dos distintas versionesuna de ellas presenta a un extremo 5` no codificante de 61 bases (para mayores detalles, consultar la sección correspondiente a datos no publicados). Sin embargo, experimentos posteriores tipo RACE dirigidos hacia el extremo 5` no generaron extremos 5` no codificantes tan extensos. Además, aparecieron variaciones en la secuencia del posible péptido señal. Esto pudiera ser consecuencia de una variabilidad entre los individuos analizados, o bien, podría tratarse de la existencia de varias copias de la secuencia que codifican para la misma proteína, copias que pudieran haber divergido entre sí en el transcurso de la evolución.

CONCLUSIÓN Y PERSPECTIVAS.

Conclusión.

En este trabajo se describe el aislamiento y caracterización de dos nuevos tipos de proteínas o péptidos contenidos en el veneno del alacrán *Pandinus imperator*: una enzima y un péptido con actividad bactericida y plasmicida.

Se purificaron las dos proteínas a partir del veneno del alacrán *Pandinus imperator* hasta llegar a niveles de homogeneidad. Se determinó tanto las secuencias peptídicas como las secuencias nucleotídicas de ambos péptidos. Se analizaron las características de las secuencias primarias y de ADNc obtenidas, comprobando las características únicas de ambos péptidos. Por una parte, la existencia de una fosfolipasa heterodimérica que está relacionada con las fosfolipasas obtenidas a partir del veneno de la abeja *Apis melifera* y del abejorro *Bombus pennsylvanicus* en el caso de la fosfolipina. Por otra parte, se mostró la presencia en el veneno del alacrán de un péptido con efecto anti-microbiano con actividad contra las fases oocineto y ooquiste de *Plasmodium berghei*, y contra bacterias que presenta una estructura híbrida entre las cecropinas y las defensinas.

Se obtuvieron las secuencias nucleotídicas de ambas proteínas, con las cuales se puede planear la expresión recombinante de ambas, con la finalidad de llevar a cabo una caracterización más completa y detallada de sus propiedades y un análisis de sus posibles aplicaciones.

Perspectivas.

Fosfolipina.

El descubrimiento de una nueva fosfolipasa tipo A2, así como su clonación, permite profundizar nuestro conocimiento acerca de las estructuras que permiten conducir la función enzimática de liberación de ácidos grasos. Además, es posible llevar a cabo estudios más detallados sobre el modo de acción de este tipo de proteínas o sobre su especificidad al substrato; usando la secuencia nucleotídica obtenida para expresar cantidades suficientes que lo permitan.

De la misma manera será posible emprender estudios de la relación estructura-función de la fosfolipina, mediante experimentos de mutagénesis dirigida.

Dada la cercanía de la secuencia peptídica entre la fosfolipina y la Imperatoxina I y las diferencias de actividad que presentan entre sí (para mayores detalles, consultar la

sección correspondiente a Datos no publicados; Fracción Ib); se pudiera analizar el papel de los aminoácidos involucrados que provocan una especificidad de substrato distinta.

Por otra parte el estudio de las estructuras secundarias de las dos fosfolipasas podría revelar nuevos patrones de plegamiento, particularmente en el caso de la fosfolipina, ya que presenta un puente disulfuro suplementario cuando se compara con la Imperatoxina I o con las fosfolipasas de *Heloderma horridum horridum.*

A nivel del estudio de la evolución de las fosfolipasas tipo A2, resulta igualmente valioso el conocimiento del cDNA de estas fosfolipasas, ya que como se mencionó anteriormente, los alacranes son animales de un antiguo origen. Un estudio filogenético más detallado de éstos, pudiera arrojar datos interesantes sobre la posible vía de evolución de las proteínas y de las enzimas.

Escorpina.

La escorpina, al igual que la fosfolipina, presenta una estructura única. Se trata de un péptido anti-microbiano que aparenta presentar una estructura híbrida entre dos clases estructurales de péptidos anti-microbianos. En un futuro cercano sería interesante investigar la importancia de cada una de sus partes estructurales y su papel antibiótico.

Asimismo, se podrá determinar con más precisión el tipo de actividad anti-bacteriana (esto es, microbicida, bacteriostática, etc.), y ampliar la gama de microorganismos ensayados, ya que se dispone de la secuencia nucleotídica que permitiría producir a la escorpina en microorganismos o en células en cultivo, a través de la ingeniería genética.

De manera paralela es posible modificar la secuencia peptídica de la escorpina, pudiendo eventualmente de esta manera aumentar su eficacia como antibiótico, aumentar su espectro anti-microbiano, o incluso mejorar su tiempo de vida media en las condiciones del ensayo.

Por otra parte, se podrán generar moscas *Drosophila* transgénicas, que sean capases de producir al péptido, con el objeto de efectuar una primera evaluación de la efectividad de la protección contra el *Plasmodium* dada por la escorpina. El paso siguiente consistiría en la realización de *Anopheles* transgénicos que expresen la escorpina de manera constitutiva, y comprobar así si esta expresión les confiere resistencia a la infección por *Plasmodium.* Estas poblaciones no serían vectores de la malaria, por lo que posiblemente interrumpirían el ciclo de reproducción del microorganismo.

Resultados añadidos en prueba.

En relación con las perspectivas futuras de este trabajo de tesis podemos mencionar (a título de prueba adicional) que parte de lo previsto en esta sección ya ha sido realizado en los laboratorios de los Drs. Lourival D. Possani, Mario Zurita y Mario Henry Rodríguez, gracias a una colaboración financiada por la Organización Mundial de la Salud y por el CONACYT. Recientemente, se obtuvo una construcción por ingeniería genética en un vector (pCASPER) que contiene al gen que codifica para la Escorpina, con el cual se pudieron obtener distintas cepas de *Drosophila* mutantes (o transgénicas) capases de expresar al péptido Escorpina. Los resultados preliminares obtenidos sostienen la idea de que las moscas transgénicas son más resistentes a la infección provocada por *Plasmodium* que las moscas silvestres utilizadas para la producción de mutantes.

Anexo I: Secuencias peptídicas de las temporinas y magaininas.

TEMPORIN A	FLPLIGRVLSGILA	MAGAININ II	GIGKFLHSAKKFGKAFVGEIMNS
TEMPORIN B	VEKRLLPIVGNLLKSLLK	MAGAININ I	GIGKFLHSAGKFGKAFVGEIMKS
TEMPORIN C	LLPILGNLLNGLLA		
TEMPORIN E	VLPIIGNLLNSLLA		
TEMPORIN F	FLPLIGKVLSGILA		
TEMPORIN G	LLPIVGNLLNSLLA		
TEMPORIN H	VEKRFFPVIGRILNG		
TEMPORIN K	LSPNLLKSLLA		
TEMPORIN L	LLPNLLKSLLA		

Anexo I. Péptidos anti-microbianos alfa helicoidales de rana. TEMPORIN L, K, F, E, (Simmaco, M. et al., 1996), TEMPORIN A, B, C, G (Simmaco, M. et al. Swissprot, 1996). Magainina II y I (Terry, A. S. et al., 1988).

Anexo II: Alineamiento de cecropinas y análogos.

```
1      WKIFKKIERVGQNVRDGIIKAGPAIQVLGTAKA
2      WKFFKKIERVGQNVRDGLIKAGPAIQVLGAAKALGK
3      WKVFKKIEKVGRNIRDGVIKAGPAIAVVGQAKA
4      WKVFKKIEKVGRHIRDGVIKAGPAITVVGQATALGK
5   GWLKKIGKKIERVGQHTRDATIQGLGVAQQAANVAATARG
6   GWLKKIGKKIERVGQHTRDATIQGLGVAQQAANVAATARG
7   GWLKKIGKKIERVGQHTRDATIQTIAVAQQAANVAATARG
8   GWLKKIGKKIERIGQHTRDATIQGLGIAQQAANVAATARG
9   GWLKKIGKKIERVGQHTRDATIQTIGVAQQAANVAATLKG
10  GWLKKIGKKIERVGQHTRDATIQGLGIAQQAANVAATARG
11  GWLKKIGKKIERIGQHTRDATIQGVGIAQQAANVAATARG
12  GWLKKIGKKIERVGQHTRDATIQGLGIAQQAANVAATARG
13  GGLKKLGKKLEGVGKRVFKASEKALPVAVGIKALG
14    RWKIFKKIEKVGQNIRDGIVKAGPAVAVVGQAATI
15     WKLFKKIEKVGRNVRDGLIKAGPAIAVIGQAKSLGK
16     WKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAKG
17     WKFFKKIEKVGQNIRDGIIKAGPAVAVVGQAASITGK
18    KWKIFKKIEKVGRNIRNGIIKAGPAVAVLGEAKAL
19     WNPFKELERAGQRVRDAVISAAPAVATVGQAAAIARG
20     WNPFKELERAGQRVRDAIISAGPAVATVGQAAAIARG
21     WNPFKELERAGQRVRDAIISAAPAVATVGQAAAIARG
22     WNPFKELERAGQRVRDAVISAAAVATVGQAAAIARGG
23    SWLSKTAKKLENSAKKRISEGIAIAIQGGPR
```

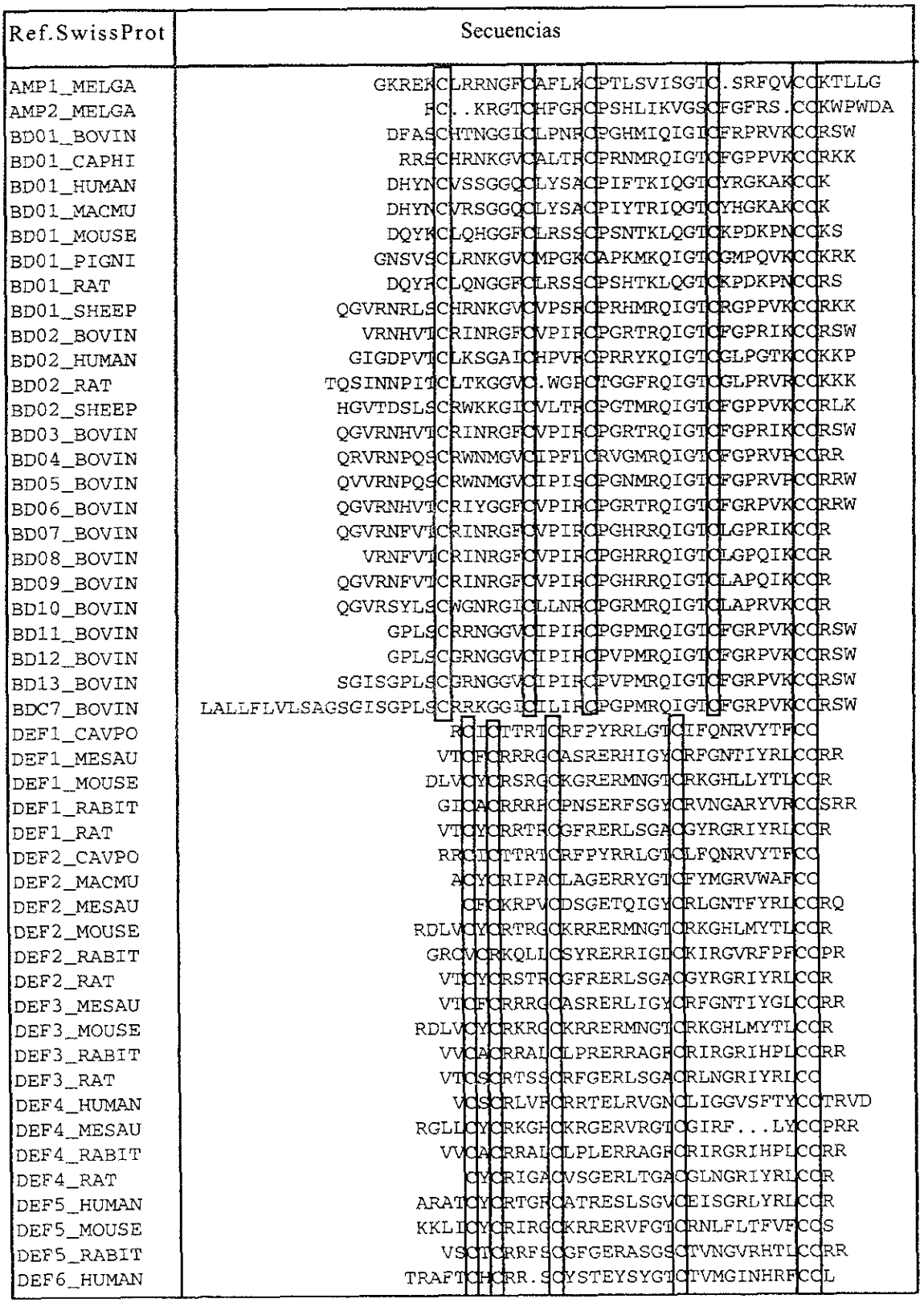
Anexo II. 1, Hyphancin IIID; 2, Hyphancin IIIE; 3, Hyphancin IIIF; 4. Hyphancin IIIG (Park S. S. et al., 1995); 5, Cecropina A1 de *Drosophila mauritiana*; 6, Cecropina A1 de *Drosophila sechellia* (Ramos-Onsins S. and Aguade M., 1998); 7, Cecropina 1 de *Ceratitis capitata* (Rosetto M. et al., 1993); 8, Cecropina A1 *Drosophila virilis* (Zhou X. et al., 1997), 9, Cecropina 2 de *Ceratitis capitata* (Rosetto M et al., 1993); 10, Cecropina 2 de *Drosophila virilis* (Zhou X. et al., 1997); 11, Cecropina 3 de *Drosophila virilis* (Zhou X. et al., 1997); 12, Cecropina A1 de *Drosophila melanogaster* (Kylsten P. et al., 1990); 13, Cecropina A de *Aedes albopictus*; 14, Bactericidina B-3 de *Manduca sexta*; 15, Cecropina A de *Bombyx mori* (Yamano Y. et al., 1994); 16, Cecropina C de *Hyalophora cecropia* (Gudmundsson G. H. et al., 1991); 17, Cecropina A de *Trichoplusia ni 22* (Kang D. et al , 1995); 18, Cecropina B de *Antheraea pernyi* (Qu X.-M. et al., 1982); 19, Bactericidina B-2 *Manduca sexta* (Dickinson L. et al., 1988); 20, Bactericidina B-3 de *Manduca sexta*; 21, Bactericidina B-4 de *Manduca sexta* , 22, Bactericidina B-5P de *Manduca sexta* (Dickinson L. et al., 1988); 23, Cecropina A1 *Sus scrofa* (Lee J.-Y. et al., 1989).

Anexo III: Alineamiento de defensinas de insectos.

Ref.Swissprot	Secuencia (Cisteinas subrayadas)
DEF4_ANDAU	GFGCPFNQGACHRHCRSI.RRRGGYCAGLFKQTCTCYR
DEF4_LEIQH	GFGCPLNQGACHRHCRSI.RRRGGYCAGFFKQTCTCYRN
DEFA_MYTED	GFGCP.NDYPCHRHCKSIPGRXGGYCGGXHRLRCTCYR
DEFB_MYTED	GFGCP.NDYPCHRHCKSIPGRYGGYCGGXHRLRCTC
DEFI_MYTGA	GFGCP.NNYQCHRHCKSIPGRCGGYCGGXHRLRCTCYRC
DEFI_AESCY	GFGCPLDQMQCHRHCQTITGRSGGYCSGPLKLTCTCYR
DEF1_STOCA	TCDLLSLWKVGHAACAAHCLVL.GDVGGYC..TKEGLCVCKE
DEF2_STOCA	TCDLLSMWNVNHSACAAHCLLL.GKSGGRC..NDDAVCVCRK
DEFA_ZOPAT	FTCDVLGFEIAGTKLNSAACGAHCLAL.GRRGGYC..NSKSVCVCR
DEFB_AEDAE	ATCDLLSGFGVGDSACAAHCIAR.GNRGGYC..NSQKVCVCRN
DEFC_AEDAE	ATCDLLSGFGVGDSACAAHCIAR.GNRGGYC..NSKKVCVCRN
DEFI_PALPR	ATCDALSFSSKWLTVNHSACAIHCLTK.GYKGGRC...VNTICNCRN
DEFI_ANOGA	ATCDLASGFGVGSSLCAAHCIAR.RYRGGYC..NSKAVCVCRN
DEFI_DROME	TCDLLSKWNWNHTACAGHCIAK.GFKGGYC..NDKAVCVCRN
DEFI_PROTE	RATCDLLSGTGINHSACAAHCLLR.GNRGGYC..NGKGVCVCRN
DEFI_TENMO	VTCDILSVEAKGVKLNDAACAAHCLFR.GRSGGYC..NGKRVCVCR
SAPB_SARPE	LTCEIDRSLCLLHCRLK.GYLRAYC..SQQKVCRCVQ
SAPC_SARPE	ATCDLLSGIGVQHSACALHCVFR.GNRGGYC..TGKGICVCRN
SAPE_SARPE	ATCDLLSGTGINHSACAAHCLLR.GNRGGYC..NGKAVCVCRN
DEFI_ALLDI	VTCDLLSFEAKGFAANHSLCAAHCLAI.GRRGGSC...ERGVCICRR
DEFI_APIME	NEERADRHRRVTCDLLSFKGQVNDSACAANCLSL.GKAGGHC...EKVGCICRKTSFKDLWDKRFG
DEFI_BOMPA	VTCDLLSIKGVAEHSACAANCLSM.GKAGGRC...ENGICLCRKTTFKELWDKRF
DEFI_PYRAP	ATCDILSFQSQWVTPNHAGCALHCVIK.GYKGGQC...KITVCHCRR

Anexo III. DEF1_STOCA, defensina 1 de *Stomoxys calcitrans* (Lehane M. J. et al., 1997); DEF4_ANDAU, defensina 4 de *Androctonus australis* (Ehret-Sabatier L., et al., 1996); DEF4_LEIQH defensina 4 de *Leiurus quinquestratus* (Cociancich S. et al., 1993); DEFA_MYTED, defensina A de *Mytilus edulis*. (Charlet M. et al., 1996); DEFA_ZOPAT, coleoptericina de *Zophobas atratus* (Bulet P. et al., 1991); DEFB_AEDAE, defensina B de *Aedes aegypti* (Lowenberger C. et al., 1995); DEFB_MYTED, defensina B de *Mytilus edulis* (Charlet M. et al., 1996); DEFC_AEDAE, defensina C de *Aedes aegypti* (Lowenberger C. et al., 1995); DEFI_AESCY, defensina de *Aeschna cyanea* (Bulet P. et al., 1992); DEFI_ALLDI, defensina de *Allomyrina dichotoma* (Miyanoshita A. et al., 1996); DEFI_ANOGA, defensina de *Anopheles gambiae* (Richmann A. M., 1996); DEFI_APIME, royalsina de *Apis melifera*; DEFI_BOMPA, defensina de *Bombus pascuorum* (Rees J. A., et al., 1997); DEFI_DROME, defensina de *Drosophila melanogaster* (Dimarcq J. L. et al., 1994); DEFI_MYTGA, defensina de *Mytilus galloprovincialis* (Hubert et al., 1996); DEFI_PALPR, defensina de *Palomena prasina* (Chernysh S. et al., 1996); DEFI_PROTE, defensina de *Phormia terranovae* (Dimarcq J.-L. et al., 1990); DEFI_PYRAP, defensina de *Pyrrhocoris apterus*. (Cociancich S. et al., 1994); DEFI_TENMO, defensina de *Tenebrio molitor* (Moon H. J. et al., 1994); SAPB_SARPE, sapecina B de células embrionarias de *Sarcophaga*. SAPC_SARPE sapecina C de células embrionarias de *Sarcophaga* (Yamada K. and Natori S., 1993); SAPE_SARPE, sapecina E (Matsuyama K. and Natori S., 1988).

Anexo IV: Alineamiento de defensinas α, β y criptidina.



Anexo IV. Secuencias de las defensinas α, ß y criptidinas. AMP1_MELGA, AMP2_MELGA, defensina 1 y 2 de *Meleagris gallopavo* (Brockus C.W. et al., 1998); BD01_BOVIN, BD02_BOVIN, ß-defensina 1 y 2 de *Bos taurus* (Selsted M. E. et al., 1993); BD01_CAPHI, ß-defensina de *Capra hircus* (Zhao C et al., 1998): BD01_HUMAN, ß-defensina 1 de *Homo sapiens sapiens* (Liu L et al., 1997); BD01_MACMU, ß-defensina 1 de *Macaca mulatta* (Kwok J. et al., 1997); BD01_MOUSE, ß-defensina 1 de *Mus musculus* (Huttner K. M. et al., 1997); BD01_RAT, ß-defensina de *Rattus norvegicus* (Jia H.P et al., 1998); BD01_SHEEP, BD02_SHEEP, ß-defensina de *Ovis aries* (Huttner K. M. et al., 1998); BD02_HUMAN, ß-defensina 2 de *Homo sapiens sapiens* (Harder J. et al., 1997), BD03_BOVIN, BDC7_BOVIN, ß-defensina 3 y ß -defensina C7 de *Bos taurus* (Tarver A. P.et al., 1998); BD05_BOVIN, BD06_BOVIN, BD07_BOVIN, BD08_BOVIN, BD09_BOVIN, BD10_BOVIN, BD11_BOVIN, BD12_BOVIN, BD13_BOVIN, ß -defensinas 6, 7, 8, 9, 10, 11, 12, 13 de *Bos taurus* (Selsted M. E. et al., 1993); DEF1_CAVPO, péptido corticostático GP-CS1 de *Cavia porcellus* (Selsted M. E. and Harwig S. S. L., 1987); DEF1_MOUSE, criptidina-1 de *Mus musculus* (Ouellette A. J. and Lualdi J. C., 1990); DEF1_RABIT, péptido microbicido NP-3A de *Oryctolagus cuniculus* (Linzmeier R. et al., 1993); DEF1_RAT, DEF2_RAT, DEF3_RAT, DEF4_RAT, péptido antibiótico de neutrófilo NP-1, NP-2, NP-3, NP-4 de *Rattus norvegicus* (Yount N. Y. et al., 1995); DEF2_CAVPO, péptido corticostático GP-CS2 de *Cavia porcellus* (Nagaoka I. et al., 1991); DEF2_MACMU, ß-defensina 2 de *Macaca mulatta* (Tang Y. Q. et al., 1999); DEF1_MESAU, DEF2_MESAU, DEF3_MESAU, DEF4_MESAU, defensina de neutrófilo 1, 2, 3, 4 de *Mesocricetus auratus* (Mak P. et. al., 1996); DEF2_MOUSE, DEF3_MOUSE, DEF5_MOUSE, CRIPTIDINA-2 , 3, 5 (Huttner K.M. et al., 1994); DEF2_RABIT, MICROBICIDAL PEPTIDE NP-3B (Zhu Q. and Solomon S., 1992); DEF3_RABIT, DEF4_RABIT, péptido anti-adrenocorticotropico III, IV (Ganz T. et al., 1989); DEF4_HUMAN, corticostatina HP-4 de *Homo sapiens sapiens* (Palfree R. G. E., Sadro L. C. and Solomon S., 1993); DEF4_MOUSE, criptidina-4 de *Mus musculus* (Ouellette A. J. et al., 1994); DEF5_HUMAN, defensina α -5 de *Homo sapiens sapiens* (Jones D. E. and Bevins C. L., 1992); DEF5_RABIT, péptido antibiótico de neutrófilo NP-4 de *Oryctolagus cuniculus* (Michaelson D. et al., 1992); DEF6_HUMAN, defensina 6 de *Homo sapiens sapiens* (Jones D. E., Bevins C. L., 1993).

Anexo V : Fosfolipasas relacionadas con la fosfolipina.

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1  MWLLREAFFFGLLAMAWAFGDEAIFEDEDIYNQALPPVPHTGITVPGTKWCGPGNTAANFEDLGR
2                    MLSRAAFLTVLLTLIYASHAAAGLSITVPGTKWCGPGNIAANYDDLGT
3                                              IIYPGTLWCGHGNKSSGPNELGR
4                                              IIYPGTLWCGHGNKSSGPNELGR
5                                              IIYPGTLWCGNGNIANGTNELGL
6                                              IIYPGTLWCGHGNVSSGPNELGR
7                                              IIYPGTLWCGHGNVSSSPDELGR
8                                                MPGTLWCGVGDSAGNSSELGV
9                                             GAFIMPGTLWCGAGNAASDYSQLGT
10                                             FLMWECTKWCGPGNNAKCESDLGP
11                                               TMWGTKWCGSGNEATDISELLG

1   ERETDKCCRAHDHCDEIIESHGALHGLPTNTDWFPILKCTCEQQFINCLQAVNSITAK TL GRI
2   EREVDTCCRAHDNCEEKIPPLEEAFGLRND.GFFPIFSCACESAFRNCLTALRNGHS LAL GKI
3   FKHTDACCRTHDMCPDVMSAGESKHGLTNTAS HTRLSCDCDDKFYDCLKNSADTISSYFVGKMY
4   FKHTDACCRTHDMCPDVMSAGESKHGLTNTAS HTRLSCDCDDKFYDCLKNSADTISSYFVGKMY
5   WKETDACCRTHDMCPDIIEAHGSKHGLTNPAD YTRLNCECDEEFRHCLHNSGDAVSAAFVGRTY
6   FKHTDACCRTHDMCPDVMSAGESKHGLTNTAS HTRLSCDCDDTFYDCLKNSGEKISSYFVGKMY
7   FKHTDSCCRSHDMCPDVMSAGESKHGLTNTAS HTRLSCDCDDKFYDCLKNSSDTISSYFVGEMY
8   FQGPDLCCREHDRCPQNISPLQYNYGIRNYRF HTISHCDCDTRFQQCLQNQHDSISDIV GVAF
9   EKDTDMCCRDHDHCENWISALEYKHGMRNYYP STISHCDCDNQFRSCLMKLKDGTADYV GQTY
10   LEADKCCRTHDHCDY IASGETKYGITNYAF FTKLNCKCEEDR  CLTEAYNKEEKESAKSST
11YWSNLDSCCRTHDHCDNIPSGQT KYGLTNE GKYTMMNCKCETAFEQCLRNVTGGMEGPAAGFVR

1  YYGSRSRCFANGHPTTGCKQYQEGTFRKRCIRYQVDKSKAKVWQFYDMPFFTIPASAG
2  YFNTKEVCFGYGHPIVSCQEKQADLFETRCLSYRVDEGQPQRWQFYDLALYTHVSGSEEDSRD
3  FNLIDTKCYKLEHPVTGCGER     TEGRCLHYTVDKSKPKVYQWFDLRKY
4  FNLIDTKCYKLEHPVTGCGER     TEGRCLHYTVDKSKPKVYQWFDLRKY
5  FTILGTQCFRLDYPIVKC  KVKSTILRECKEYEFDTNAPQKYQWFDVLSY
6  FNLIDTKCYKLEHPVTGCGER     TEGRCLRYTVDKSKPKVYQWFDLRKY
7  FNILDTKCYKLEHPVTGCGKR     TEGRCLNYTVDKSKPKVYQWFDLRKY
8  FNVLEIPCFVLE EQEACVAWY
9  FNVLKIPCFELE EGEGCVDWNFWLE   CTESKIMPVAKLVSAAPYQAQAETQSGEG
10KRLQNFYFGTYSP    ECYVVTCNS DAGCENGVATWKKSYKD
11      KTYFDLY  GNGCYNQCPSQ SEECPDGVATYTGEAGYGAWAINLKG
```

Anexo V. 1, secuencia deducida a partir del gen de la fosfolipasa A2 CG11124 de *Drosophila melanogaster* (Adams, M. D. et al., 2000)(en itálicas se muestra al péptido señal); 2, secuencia deducida a partir del gen de la fosfolipasa A2 CG1672 de *Drosophila melanogaster* (Adams, M. D. et al., 2000)(en itálicas se muestra al péptido señal); 3, fosfolipasa A2 de abeja (*Apis Mellifera*) (Kuchler, K., et al., 1989); 4, secuencia deducida del cDNA de la fosfolipasa de abeja (*Apis mellifera*) (Scott, D. L., et al., 1990); 5, fosfolipasa A2 del abejorro (*Bombus pennsylvanicus*) (Hoffman, D. R., 1995); 6, fosfolipasa A2 de *Apis cerana*; (Hoffman, D. R. and Schmidt, J. O., 1999); 7, fosfolipasa A2 de *Apis dorsata* (Hoffman, D. R., Schmidt, J. O., 1999); 8, fosfolipasa A2 del grupo III secretada por el ser humano (*Homo sapiens sapiens*) (Valentin, E , et al., 2000); 9, fosfolipasa A2 del monstruo de Gila (*Heloderma suspectum*) (Gomez, F., et al., 1989); 10, fosfolipina (subrayada la secuencia correspondiente a la subunidad pequeña); 11, imperatoxina I (Zamudio F. et al., 1997) (subrayada la secuencia correspondiente a la subunidad pequeña).

Anexo VI: Zamudio, F., Conde, R., Arevalo, C., Becerril, B., Brian, M., Valdivia, H., Possani, L., (1997). The mechanism of inhibition of Ryanodine Receptor Channels by Imperatoxin I, a heterodimeric protein from the scorpion Pandinus imperator. *J. Biol. Chem.* **272** (18): 11886-11894.

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The Mechanism of Inhibition of Ryanodine Receptor Channels by Imperatoxin I, a Heterodimeric Protein from the Scorpion *Pandinus imperator**

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From the §Department of Physiology, University of Wisconsin Medical School, Madison, Wisconsin 53706, the ‡Department of Molecular Recognition and Structural Biology, Biotechnology Institute, National Autonomous University of Mexico, Cuernavaca, Morelos 62271, Mexico, and the ¶National Institute of Mental Health, Unit on Molecular Structures, Bethesda, Maryland 20892

We present an in-depth analysis of the structural and functional properties of Imperatoxin I ($IpTx_i$), an ~15-kDa protein from the venom of the scorpion *Pandinus imperator* that inhibits Ca^{2+} release channel/ryanodine receptor (RyR) activity (Valdivia, H. H., Kirby, M. S., Lederer, W. J., and Coronado, R. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 12185–12189). A cDNA library was prepared from the venomous glands of this scorpion and used to clone the gene encoding $IpTx_i$. From a single continuous messenger RNA, the information coding for the toxin is translated into two mature polypeptide subunits after elimination of a basic pentapeptide. The $IpTx_i$ dimer consists of a large subunit (104-amino acid residues) with phospholipase A_2 (PLA_2) activity covalently linked by a disulfide bond to a smaller (27 amino acid residues), structurally unrelated subunit. Thus, $IpTx_i$ is a heterodimeric protein with lipolytic action, a property that is only shared with β-bungarotoxins, a group of neurotoxins from snake venoms. The enzymatic subunit of $IpTx_i$ is highly homologous to PLA_2 from bee (*Apis mellifera*) and lizard (*Heloderma horridum*) venoms. The small subunit has no significant similarity to any other known peptide, including members of the Kunitz protease inhibitors superfamily that target the lipolytic effect of β-bungarotoxins. A synthetic peptide with amino acid sequence identical to that of the small subunit failed to inhibit RyR. On the other hand, treatment of $IpTx_i$ with *p*-bromophenacylbromide, a specific inhibitor of PLA_2 activity, greatly reduced the capacity of $IpTx_i$ to inhibit RyRs. These results suggested that a lipid product of PLA_2 activity, more than a direct $IpTx_i$-RyR interaction, was responsible for RyR inhibition.

Scorpion venoms contain families of small basic proteins that modify the gating mechanism of Na^+ channels or block with high affinity K^+ channels of excitable cells (1, 2). These toxins have been invaluable tools in the identification, purification, structural mapping, and functional characterization of the corresponding ionic channels. The study of other ionic channels have also been aided by toxins from poisonous animals. For instance, snake venoms contain potent neurotoxins that block acetylcholine receptors (3), and snail and spider venoms contain small molecular weight proteins directed against neuronal Ca^{2+} channels (4, 5). A Cl^- channel-specific blocker peptide was also recently isolated from a scorpion venom (6). Thus, there exist a vast array of natural ligands useful for structural and functional characterization of ionic channels.

The Ca^{2-} release channel of SR[1] constitutes the major pathway for Ca^{2-} release during the process of excitation-contraction coupling in cardiac and skeletal muscle (7). The Ca^{2+} release channel binds the plant alkaloid ryanodine with nanomolar affinity, hence the name ryanodine receptor (RyR). Ryanodine has been an invaluable tool in the structural and functional characterization of RyR. The alkaloid binds to a conformationally sensitive domain on the RyR protein and may be used in binding assays as an index of the functional state of the channel (8, 9). However, ryanodine displays extremely slow dissociation kinetics that make its effect practically irreversible. Furthermore, certain concentrations of ryanodine may open RyRs while others may block them (10), leading to ambiguous results.

From the venom of the African scorpion *Pandinus imperator*, we isolated Imperatoxin I ($IpTx_i$), a ~15-kDa protein that inhibited [^{3}H]ryanodine binding to cardiac and skeletal SR by blocking RyR channels (11). At concentrations well above the half-maximal effective concentrations (ED_{50}) exhibited for RyR, $IpTx_i$ did not modify the binding of ligands targeted against other transporters and ionic channels of striated muscle (11). $IpTx_i$ blocked RyR rapidly and reversibly, and when injected in ventricular cells it decreased twitch amplitude and intracellular Ca^{2+} transients, suggesting a selective blockade of Ca^{2+} release from the SR (11).

In this study, we carried out an in-depth analysis of the mechanism of action of $IpTx_i$ on RyRs of cardiac and skeletal muscle. We determined the complete amino acid and nucleotide sequence of $IpTx_i$ and show that, like β-bungarotoxins, $IpTx_i$ is a heterodimeric protein composed of a high molecular weight subunit with PLA_2 activity and a small, structurally unrelated

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; *p*BPB, *p*-bromophenacyl bromide; PLA_2, phospholipase A_2; RyR, ryanodine receptor; lyso-PC, lysophosphatidylcholine; HPLC, high performance liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.

subunit. We also show that free fatty acids, lipid products of $IpTx_i$-PLA_2 activity, are involved in the inhibition of RyR. $IpTx_i$ thus offers an alternative way to block RyR distinct from ryanodine and other ligands that require a physical interaction with the RyR protein.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Protease lysine C (Lys-C) and restriction enzymes were from Boehringer Mannheim. Chemicals and solvents for peptide sequencing were from Millipore Co. Primers for polymerase chain reaction (lambda gt11 forward and reverse; 1218 and 1222) and Vent^R polymerase were from New England BioLabs. DNA sequencing kit (Sequenase version II), was from U. S. Biochemical Corp. Subcloning and sequencing vector (pBluescript phagemid; pKS), M13 −20, and M13 reverse sequencing primers were from Stratagene. Brain phosphatidylethanolamine and brain phosphatidylserine were from Avanti Polar Lipids. [^{3}H]Ryanodine was from DuPont NEN. *p*-Bromophenacyl bromide (*p*BPB) was from Sigma.

Purification of $IpTx_i$—P. imperator venom was obtained by electric stimulation of scorpions maintained alive in the laboratory. Venom (120 mg per batch) was suspended in double distilled water and centrifuged at 15,000 × *g* for 30 min. The supernatant was applied onto a column (0.9 × 190 cm) of Sephadex G-50 superfine (Pharmacia Biotech Inc.). Fractions were eluted with 20 mM NH_3OAc (pH 4.7) at a flow rate of 20 ml/h. Fraction II containing $IpTx_i$ was applied to a column (0.9 × 30 cm) of carboxymethyl (CM)-cellulose 32 (Whatman) equilibrated with 20 mM NH_3OAc (pH 4.7). Peptides were eluted at a flow rate of 20 ml/h with a linear gradient of 250 ml of 20 mM NH_3OAc (pH 4.7) and 250 ml of the same buffer containing 0.55 M NaCl. Peptides displaying capacity to inhibit [^{3}H]ryanodine binding and phospholipase activity were dialyzed against deionized water (3 × 30 min), concentrated by lyophilization, and injected into a C_4 reverse-phase HPLC column (Vydac). $IpTx_i$ was eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid running at 1 ml/min for 60 min. The purity and identity of $IpTx_i$ was confirmed by amino acid sequence analysis, as described for Na^+ channel-blocking peptides (12). $IpTx_i$ was quantified either by amino acid analysis or by absorbance at 280 nm ($A_{280\ nm}$) using an extinction coefficient (ϵ) = 14,952.

Amino Acid Analysis and Microsequencing of $IpTx_i$—Amino acid analysis was performed on samples hydrolyzed in 6 N HCl with 0.5% phenol at 110 °C in evacuated, sealed tubes as described (13). Reduction of $IpTx_i$ with dithiothreitol and alkylation with iodoacetic acid was performed as described (13). Reduced and alkylated $IpTx_i$ was cleaved with protease Lys-C in 200 μl of 25 mM Tris-HCl (pH 7.2), and 1.0 mM EDTA, at a enzyme:peptide ratio = 1:100. Microsequence determination of native, reduced, and carboxymethylated toxin, and their peptide fragments was carried out with a 6400/6600 MilliGen/Biosearch Prosequencer, using the peptide adsorbed protocol on CD Immobilon membranes.

Cloning and Sequencing of the cDNA Encoding $IpTx_i$—Two oligonucleotides encoding for two different regions of $IpTx_i$ were synthesized as described (13). Oligonucleotide 1 (5′-AC(*N*)ATGTGGGG(*N*)AC(*N*)AA(G/A)TGGTG-3′; where *N* means any nucleotide) encoded for the first 8 amino acids of the amino terminus of the large subunit of $IpTx_i$. Oligonucleotide 2 (5′-GA(G/A)GC(*N*)GG(*N*)TA(T/C)GG(*N*)GC(*N*)TGGGC-3′) encoded for residues 14–21 of the small subunit. Total RNA was purified from the venomous glands (telsons) of 30 scorpions. The telsons were pulverized in liquid nitrogen and poured into 10 ml of GT buffer (4 M guanidinium isothiocyanate, 0.025 M sodium citrate (pH 7.0), 0.5% *N*-lauryl sarcosine, and 0.03 M β-mercaptoethanol), vortexed, and centrifuged at 5,000 × *g* for 10 min. One ml of 2 M sodium acetate (pH 4) was added to the recovered supernatant. The resulting solution was extracted with 10 ml of water-saturated phenol plus 2 ml of chloroform isoamyl alcohol (49:1, v:v), allowing to sit on ice for 15 min. After centrifuging at 10,000 × *g* for 20 min at 4 °C, the aqueous layer was precipitated with 1 volume of isopropyl alcohol, incubated at −20 °C for 1 h, and centrifuged at 10,000 × *g* for 20 min at 4 °C. The pellet was dissolved in 0.3 ml of GT buffer and 0.3 ml of isopropyl alcohol, incubated at −20 °C for 1 h, and pelleted again. The pellet was washed with 95% ethanol, briefly dried under vacuum, and resuspended in RNase-free water. Messenger RNA was purified following the instructions of the Hybond-mAP protocol (messenger affinity paper, Amersham Corp., RPN 1511). cDNA synthesis was performed as described (13) from 5 μg of mRNA. The cloning of the cDNA library was also performed as described (13). The screening of the cDNA library was performed with oligonucleotides 1 and 2 separately, but the clones detected with oligonucleotide 1 were analyzed first. Inserts of cDNA from positive clones were polymerase chain reaction-amplified using λ gt11 forward and reverse primers. Polymerase chain reaction products were purified from agarose gel and ligated into the *Eco*RV site of pBluescript (pKS) phagemid. The ligation reactions were used to transform *Escherichia coli* DH5-α cells. Plasmid DNA from white colonies was digested with *Bam*HI and *Hin*dIII to verify the size of the original inserts. Clones of interest were sequenced using the Sequenase^R kit version 2 (U. S. Biochemical Corp.) on both strands. Oligonucleotides λ gt11 forward, λ gt11 reverse, M13 -20, and M13 reverse were used for sequencing.

Sequence Comparisons—The amino acid sequence of the large and small subunits of $IpTx_i$ were compared with those of other proteins deposited in the protein data base of GenBank (Los Alamos National Laboratory, Los Alamos, NM) by computer analysis using the program Blitz version 1.5 (Biocomputing Research Unit, University of Edinburgh, UK).

Determination of Phospholipase A_2 Activity—Phospholipase A_2 activity was determined by the titration method of Shiloah *et al.* (14), using dilute egg yolk (1:20 in saline solution) as substrate. Liberation of acid was measured at pH 8.0 and 37 °C by titration with 5 mM NaOH under a constant stream of N_2. One unit of enzyme is defined as the amount of enzyme that liberates 1 μmol of free fatty acid/min under the above conditions. Inhibition of phospholipase A_2 with *p*BPB was carried out as described by Díaz *et al.* (15). One hundred μl of 200 μM $IpTx_i$ in 35 mM Tris (pH 8.0) were mixed with 10 μl of 2 mM *p*-BPB in acetone and incubated for 16 h at room temperature under dim light. The reaction was stopped by filtration in a Sephadex G-10 column. *p*BPB-treated $IpTx_i$ elutes in the void volume of the column, whereas the excess of *p*BPB is retarded.

Synthesis of the Small Subunit of $IpTx_i$—The small subunit of $IpTx_i$ was synthesized by the solid phase method of Merrifield (16), using *t*-butyloxycarbonyl-amino acids. This subunit contains a Cys residue at position 4. To avoid formation of inter-peptide disulfide bonds, Cys^4 was substituted by Ala (peptide A), Met (peptide M), or Cys (peptide C) protected with 3-nitro-2-pyridinesulfonyl (thiol-protecting group that will not allow formation of disulfide bonds). At the end of the synthesis all three peptides were separated in a C_{18} reverse-phase HPLC column, using the conditions described above. The purity of the synthetic peptides was confirmed by both amino acid analysis and microsequencing, as described above.

[^{3}H]Ryanodine Binding Assays—[^{3}H]Ryanodine binding to pig cardiac and rabbit skeletal SR vesicles was carried out for 90 min at 36 °C in 0.1 ml of 0.2 M KCl, 1 mM Na_2EGTA, 0.995 mM $CaCl_2$, 10 mM Na-Pipes (pH 7.2). The calculated free Ca^{2+} was 10 μM. [^{3}H]Ryanodine (68.4 Ci/mmol) was diluted directly in the incubation medium to a final concentration of 7 nM. Protein concentration was 0.2–0.4 and 0.3–0.5 mg/ml for skeletal and cardiac SR, respectively. Samples were filtered on Whatman GF/B glass fiber filters and washed twice with 5 ml of distilled water. A Brandel M24R cell harvester was used for filtration. Nonspecific binding was determined in the presence of 10 μM unlabeled ryanodine and has been subtracted from each sample.

Planar Bilayer Technique—Recording of single RyR in lipid bilayers was performed as described previously (17). Briefly, a phospholipid bilayer of phosphatidylethanolamine:phosphatidylserine (1:1 dissolved in *n*-decane to 20 mg/ml) was formed across an aperture of ~300 μm diameter in a delrin cup. The cis chamber (900 μl) was the voltage control side connected to the head stage of a 200 A Axopatch amplifier, and the trans chamber (800 μl) was held at virtual ground. Both chambers were initially filled with 50 mM cesium methane sulfonate and 10 mM Tris/Hepes (pH 7.2). After bilayer formation, cesium methane sulfonate was raised to 300 mM in the cis side, and 100–200 μg of SR vesicles were added. After detection of channel openings, Cs^+ in the trans chamber was raised to 300 mM to collapse the chemical gradient. Single channel data were collected at steady voltages (−30 mV) for 2–5 min. Channel activity was recorded with a 16-bit VCR-based acquisition and storage system at a 10-kHz sampling rate. Signals were analyzed after filtering with an 8-pole Bessel filter at a sampling frequency of 1.5–2 kHz. Data acquisition and analysis were done with Axon Instruments software and hardware (pClamp v6.0, Digidata 200 AD/DA interface).

RESULTS

Purification of $IpTx_i$—Purification of $IpTx_i$ from *P. imperator* venom was performed in three chromatographic steps as described under "Experimental Procedures" and shown in Fig. 1. After fractionation of whole venom in Sephadex G-50 (Fig. 1*A*), five fractions were collected and assayed for effects on [^{3}H]ryanodine binding. Fraction 2 contained polypeptides in the

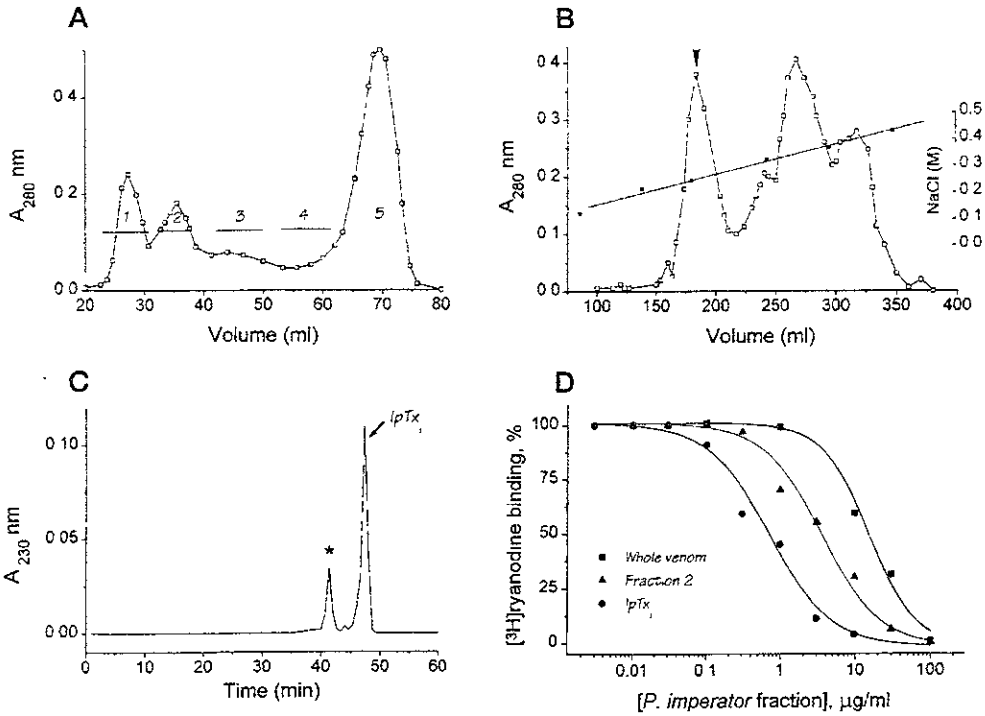

FIG. 1. **Purification of IpTx$_i$.** *A*, *P. imperator* soluble venom (120 mg of protein) was applied to a Sephadex G-50 column (0.9 × 190 cm) equilibrated and run with 20 mM ammonium acetate buffer (pH 4.7). 5-ml samples were collected and tested for their capacity to inhibit [^{3}H]ryanodine binding. Only fraction 2 totally inhibited [^{3}H]ryanodine binding. *B*, fraction 2 was further separated through a CM-cellulose column (0.9 × 30 cm), equilibrated, and run with 20 mM ammonium acetate buffer (pH 4.7). A linear gradient of sodium chloride resolved 5 subfractions, the first of which (indicated by the *arrow*) contained IpTx$_i$. *C*, the subfraction from the CM-cellulose column containing IpTx$_i$ was lyophilized and injected into a C_4 reverse-phase HPLC column and eluted with a 0–100% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The peak labeled with an *asterisk* was not studied further. *D*, dose-response curve for whole venom and purified components. [^{3}H]Ryanodine (7 nM) was incubated with cardiac SR protein in 0.2 M KCl, 10 μM $CaCl_2$, 10 mM Na-Hepes (pH 7.2) in the absence (control, 100%) and the presence of indicated concentrations of venom components. Nonspecific binding was determined in the presence of 20 μM ryanodine and has been subtracted from this and subsequent results.

range of 8–16 kDa and was the only fraction that inhibited [^{3}H]ryanodine binding. The second step in the purification of IpTx$_i$ consisted of ion-exchange chromatography in CM-cellulose (Fig. 1*B*). The fraction containing IpTx$_i$ (indicated by the *arrow*) eluted early in the run, when the concentration of NaCl was ~160 mM. Fig. 1*C* shows the chromatographic profile of IpTx$_i$ after elution from a reverse-phase C_4 HPLC column. Only a minor contaminant was present (labeled with *asterisk*), which was discarded for further studies of IpTx$_i$ structure. However, amino acid sequence analysis of purified IpTx$_i$ yielded two different amino acids per reaction cycle, with an apparent equivalent stoichiometry. This indicated either that a contaminant was still present at the end of our purification procedure or that IpTx$_i$ was composed of two different peptide subunits.

We determined the dose-response relationship for each of the active venom components to assess their potency to inhibit RyRs (Fig. 1*D*). Whole *P. imperator* venom and fraction 2 inhibited [^{3}H]ryanodine binding to *cardiac* SR with a concentration of 20.2 and 4.3 μg/ml, respectively, yielding the half-maximal effect (IC_{50}). IC_{50} for pure IpTx$_i$ was 0.7 μg/ml. Based on this value and the molecular mass of IpTx$_i$ (~15 kDa), the estimated apparent dissociation constant (K_d) was 46 nM. Essentially identical results were obtained when *skeletal* SR was used for displacement studies (not shown).

Electrophoretic Analysis of IpTx$_i$—To elucidate whether IpTx$_i$ was composed of two subunits, an aliquot of IpTx$_i$ was reduced and alkylated. The modified toxin was then chromatographed in a Bio-Gel P30 column (Fig. 2*A*) to eliminate reaction by-products and excess of reagents. Three peaks were obtained (Fig. 2*A*) and analyzed by SDS-PAGE (Fig. 2*B*). Peak 1 (labeled *Red*, abbreviation for reduced) had lower molecular mass than native IpTx$_i$ (labeled *Nat*); peak 2 could not be resolved in the same gel (not shown), but direct sequencing indicated that it corresponds to a ~3-kDa peptide (Fig. 3*A*); peak 3 was not peptidic in nature, indicating that it corresponded to the reducing and alkylating reagents. Fig. 2*C* shows that the apparent molecular mass of native IpTx$_i$ was ~15 kDa and that of the reduced IpTx$_i$ was ~12 kDa. Thus, treatment of IpTx$_i$ with thiol-reducing agents separates a large (~12 kDa) from a small (~3 kDa) subunit.

Peptide Sequence Determination—The reduced and alkylated derivatives of IpTx$_i$ (peak 1 and 2 of Fig. 2*A*) were sequenced by direct Edman degradation. As shown in Fig. 3*A*, we identified the first 27 amino acid residues of the large subunit, and all 28 amino acid residues of the small subunit. To identify the remaining residues, the large subunit was cleaved with Lys-C endopeptidase, which yielded three peptide fragments that eluted with different retention times in an HPLC column (data not shown). The amino acid sequence of each of these peptides, obtained also by Edman degradation, is *underlined* in Fig. 3*A*.

Cloning and Sequencing of the cDNA Encoding IpTx$_i$—Two oligodeoxynucleotides synthesized according to the amino acid sequence of stretch 1–8 of the large subunit and stretch 14–21 of the small subunit were used to screen a cDNA library, constructed from the venomous glands of *P. imperator* scorpi-

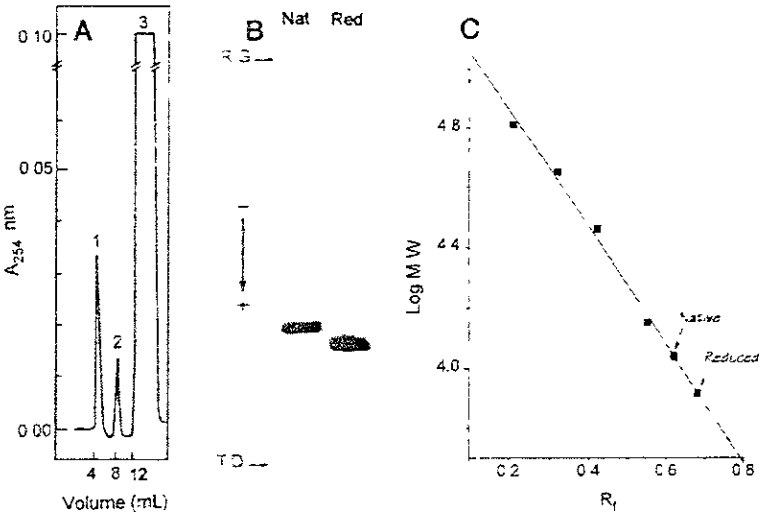

FIG. 2. **Separation of large and small subunits of IpTx$_i$.** *A*, IpTx$_i$ (100 μg) was reduced and alkylated as described under "Experimental Procedures," applied to a Bio-Gel P30 column (0.9 × 27 cm), and eluted with 10% acetic acid. Peak 1 corresponds to a ~12-kDa peptide, peak 2 to a ~3-kDa peptide (determined by direct sequencing), and peak 3 to reaction by-products. *B*, SDS-PAGE analysis of 3 μg of native IpTx$_i$ (*Nat*) and 3 μg of peak 1 (*Red*). Gel was 10% polyacrylamide. Proteins were stained with Coomassie Blue. The running gel (*R.G.*) and the tracking dye (*T.D.*) positions are indicated by *arrows*. *C*, the retention factor (R_f) for various molecular weight markers is plotted as a function of $\log_{10}$ molecular weight (*M.W.*). The apparent molecular weight for native and reduced IpTx$_i$ was extrapolated from a linear regression to the data points.

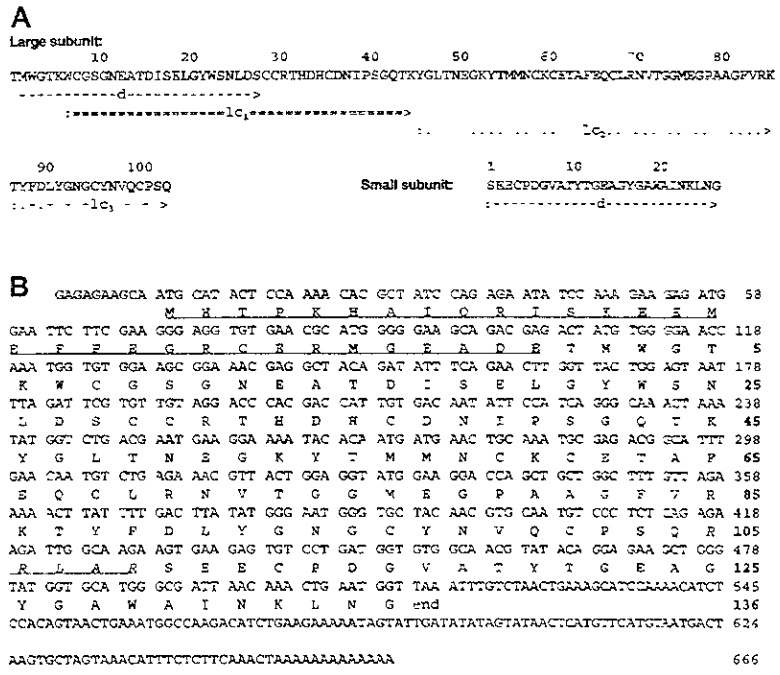

FIG 3. **Amino acid and nucleotide sequences of IpTx$_i$.** *A*, the complete amino acid sequence of IpTx$_i$ was obtained from direct Edman degradation using peak 1 (large subunit) and peak 2 (small subunit) of Fig. 2*A* (labeled *d*) and from proteolytic fragments (lc_1, lc_2, and lc_3) of peak 1. To obtain the proteolytic fragments, peak 1 (100 μg) was incubated for 4 min at 36 °C with 1 μg of Lys-C endopeptidase in Tris buffer as specified in the Boehringer catalog. The reaction products were separated by HPLC, essentially as described in the legend to Fig. 1*A*. *Numbers on top* of the amino acid sequence correspond to the amino acid positions in the primary structure. *B*, nucleotide sequence of the cDNA gene encoding for the entire IpTx$_i$ molecule with the deduced amino acid sequence below each base triplet. The putative signal peptide sequence (relative position −31 to −1) is *underlined*. The pentapeptide *RRLAR* (relative position 105–109) is not found in the mature protein and is assumed to be cleaved during processing. *Numbers on right* refers to the nucleotide and amino acid positions.

ons. These oligodeoxynucleotides were used as probes to isolate a full-length IpTx$_i$ cDNA clone. Both probes led to the isolation of the same cDNA clone. Its complete nucleotide sequence is shown in Fig. 3*B*. This sequence contained an open reading frame of 167 amino acids encompassing 1) a putative signal peptide (first underlined 31 amino acids, positions −31 to −1); 2) the mature ~12-kDa, large subunit of IpTx$_i$ (104 amino acids, positions 1–104); 3) a putative connector pentapeptide (*RRLAR*, positions 105–109); and 4) the mature small subunit (27 amino acids; positions 110–136). Thus, a single continuous cDNA clone encoded the two polypeptide subunits of IpTx$_i$. This finding confirmed that IpTx$_i$ is a heterodimeric protein. Since treatment of IpTx$_i$ with thiol-reducing agents (Fig. 2*A*) effectively separates both subunits, this suggests that they are covalently linked by a disulfide bridge.

Sequence Homology of IpTx$_i$ Subunits—A comparison of the amino acid sequence of the two subunits of IpTx$_i$ with sequences available from GenBank revealed intriguing results. First, the small subunit shared no significant similarity with sequences of scorpion peptide blockers of Na^+, K^+, or Cl^- channels, with members of the Kunitz protease inhibitor superfamily, or with any other sequence deposited in the protein data base. Thus, this small subunit constitutes a new class of scorpion peptides. On the other hand, Fig. 4 shows that the large subunit of IpTx$_i$ was 38% homologous to PLA_2 from honey bee (*A. mellifera*) venom and 35% homologous to PLA_2 from heloderma (*H. horridum*) venom. These two PLA_2s compose group III of secreted PLA_2 (18), whose main features are low molecular mass (~14 kDa), high disulfide bond content, and strict dependence on Ca^{2+} for lipolytic effect. Since the major criterion for classification is sequence homology more than function, IpTx$_i$ may be classified within this group with the remarkable difference, however, of possessing an accessory protein.

```
-3  1                                                    50
    TMWGTKWCGSGNEATDISELGYWSNLDSCCRTHDHC-DNIPS-GQTKYGL
      IIYPGTLWCGHGNKSSGPNELGRFKHTDACCRTHDMCPDYM-SAGESKHGL
  GAFIMPGTLWCGAGNAASDYSQLGTEKDTDMCCRDHDHC-ENWISALEYKHGW

    51                                                   100
    TNEGKYTMMNCKCETAFEQCLRNVTGGMEGPAAGFVRKTYFD-LYGNGCY
    TNTASHTRLSCDCDDKFYDCLKNSADTISSY---PVGMYFN-LIDTKCY
    RNYYPSTISHCDCDNQFRSCLMKLSDGTADY----VGQTYFNVLKIP-CF

    101                                                  153
    NVQCPSQ  .....  .. ..  ......  . . ..  .. ........
    KLEHPVTGCGERTEGR-CLHYTVDKSKPR-VYQWFDLRKYXXNSRG-GSX
    SLEEGS-GCVDWNFWLECTESKIMPVAKLVSAAPYQAQAETQSGEG ...

    151
    ... ...  (IpTx$_i$)
    EYHFEIVY (Bee phospholipase)
    .... . . (Heloderma phospholipase)
```

FIG 4 **Amino acid sequence homology between the large subunit of IpTx$_i$ and PLA$_2$s from group III.** The amino acid sequence of the large subunit of IpTx$_i$ (*first row*) was aligned with that of bee (*A. mellifera, second row*) and lizard (*H. suspectum, third row*) venom PLA$_2$. A few gaps (*dashes*) were introduced to enhance similarities. *Bold letters* indicate exact homology for the three PLA$_2$s. *Dots* indicate end of the sequence, whereas *X* indicates unknown residues, as indicated in the reference source. Amino acid sequences taken from IpTx$_i$, this work, bee PLA$_2$, GeneBank Accession No X16709; *Heloderma suspectum* PLA$_2$, Gomez *et al* (32).

Lack of Inhibition by the Short Subunit of IpTx$_i$—We tested if the small subunit of IpTx$_i$ was responsible for blocking RyR. Reduced and carboxymethylated small subunit (peak 2 of Fig. 2*A*), at concentrations up to 15 μM, had no effect on the binding of [^{3}H]ryanodine to cardiac or skeletal RyR (data not shown). To discard the presence of spurious reagents as being the cause of negative results, we synthesized the small subunit in three distinctive forms as follows: peptide C was similar to the native small subunit, and peptides A and M were also similar to the native small subunit, except that Cys^4 was substituted by Ala and Met, respectively. This avoided the formation of interpeptide disulfide bridges caused by oxidation of Cys^4. The three peptides were HPLC-purified before testing. Table I shows that none of the purified synthetic peptides had significant effect on the binding of [^{3}H]ryanodine to cardiac RyR or on the binding of [^{3}H]PN200–110 to voltage-dependent Ca^{2+} channels of sarcolemma (19).

The Phospholipase A$_2$ Activity of IpTx$_i$ Is Important to Inhibit RyR—We next investigated if the large subunit of IpTx$_i$, or its enzymatic activity, was involved in RyR inhibition. The reduced and carboxymethylated form of the large subunit of IpTx$_i$ (peak 1) was incapable of inhibiting [^{3}H]ryanodine binding to cardiac or skeletal RyR. However, this was expected given that internal disulfide bridges are essential for preservation of the toxin's tridimensional structure as well as for expression of its enzymatic activity (20). We thus resorted to *p*BPB, a covalent modifier of His residues that specifically blocks phospholipase A$_2$ activity (15) without affecting disulfide bonds. The rate of fatty acid liberation by 1 mol of IpTx$_i$ was 384 ± 26 and 41 ± 12 mol min^{-1} in control and after incubation with *p*BPB, respectively (n = 4; data not shown). Thus, treatment of IpTx$_i$ with *p*BPB decreased substantially its PLA$_2$ activity. Fig. 5 shows that *p*BPB-treated IpTx$_i$ decreased dramatically its capacity to inhibit the binding of [^{3}H]ryanodine to cardiac and skeletal RyR (*open symbols*). This was in contrast to control IpTx$_i$ (a batch of IpTx$_i$ that underwent the same treatment as *p*BPB-treated IpTx$_i$, except that *p*BPB was omitted), which retained its capacity to inhibit cardiac and skeletal RyR with high affinity (*filled symbols*).

Ca^{2+} Dependence of IpTx$_i$ Effect—The enzymatic activity of PLA$_2$ from group III requires Ca^{2+} for catalysis (20). Therefore, we expected that if the inhibition of RyRs by IpTx$_i$ resided in its enzymatic activity, inhibition should be apparent only in the presence of micromolar [Ca^{2+}] Fig 6 shows that Ca^{2+} was essential for binding of [^{3}H]ryanodine to RyRs and for detection of the IpTx$_i$ effect The *left panel* shows the Ca^{2+} dependence of [^{3}H]ryanodine binding to *skeletal* SR and the effect of IpTx$_i$. Specific binding in control (*open circles*) had a threshold for detection at 100 nM [Ca^{2+}] (*p*Ca 7) and was optimal at 10–100 μM [Ca^{2+}]. Higher [Ca^{2+}] inhibited binding, giving rise to a *bell-shaped curve*. In the presence of IpTx$_i$ (*filled circles*), the binding curve was dramatically decreased in absolute values. The percentage of IpTx$_i$ inhibition was 5 5, 32, and 71% at *p*Ca 7, 6, and 5, respectively. No further inhibition was observed at higher [Ca^{2+}]. Thus, the degree of IpTx$_i$ inhibition increased with [Ca^{2+}].

In *cardiac* SR (Fig. 6, *right panel*), the Ca^{2+} dependence of [^{3}H]ryanodine binding also had a threshold for detection at *p*Ca 7 and a maximum at *p*Ca 5 (control, *open circles*). However, unlike skeletal SR, inactivation of binding by high [Ca^{2+}] was not pronounced Binding decreased only by 30% with respect to maximum in cardiac RyRs, and it decreased by 80% in skeletal RyRs. This distinctive response of RyR to Ca^{2+} is also displayed by individual RyR reconstituted in lipid bilayers (8, 21), indicating that the binding assay effectively tracks the activity of the receptor In the presence of IpTx$_i$, the binding curve was markedly decreased The percentage of inhibition was 10, 38, and 83% at *p*Ca 7, 6, and 5, respectively. Thus, although cardiac and skeletal RyRs respond differently to Ca^{2+}, the inhibitory effect of IpTx$_i$ increased equally with [Ca^{2+}] in both isoforms

Inhibition of RyR by Supernatant of IpTx$_i$-treated SR Vesi-

TABLE I

Effect of synthetic peptide analogs of the small subunit of IpTx$_i$ on the binding of [^{3}H]ryanodine and [^{3}H]PN200–110 to cardiac sarcolemma and SR vesicles

Peptide concentration tested was 10 μM. Results are the mean ± S D. of three independent determinations

	[^{3}H]Ryanodine binding[a]	[^{3}H]PN200–110 binding[b]
	c_1	c_2
Control	100	100
Peptide A	116 ± 8	100 ± 6
Peptide M	110 ± 4	111 ± 12
Peptide C	106 ± 5	111 ± 7

[a] Binding of [^{3}H]ryanodine to SR vesicles was carried out as described in the text.

[b] Binding of [^{3}H]PN200–110 to cardiac sarcolemma was performed as described (19).

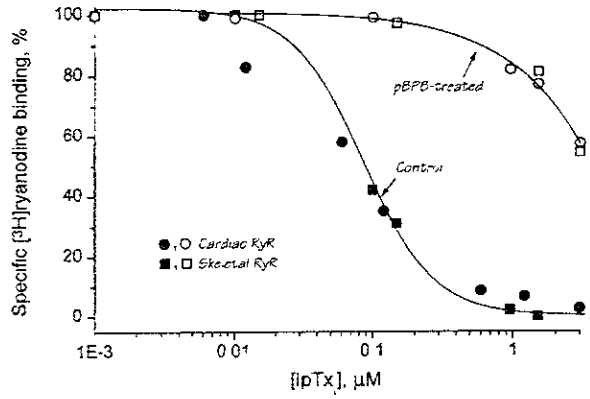

FIG 5. **Treatment with a PLA$_2$ inhibitor lowers the potency of IpTx$_i$ to inhibit RyR.** IpTx$_i$ (200 μM) was incubated with 2 mM *p*BPB (*filled symbols*) for 16 h as described under "Experimental Procedures" or with acetone (*open symbols*), the drug vehicle, as control Binding of [^{3}H]ryanodine to cardiac (*circles*) or skeletal (*squares*) SR was performed in the absence (defined as 100%) or in the presence of indicated concentrations of IpTx$_i$

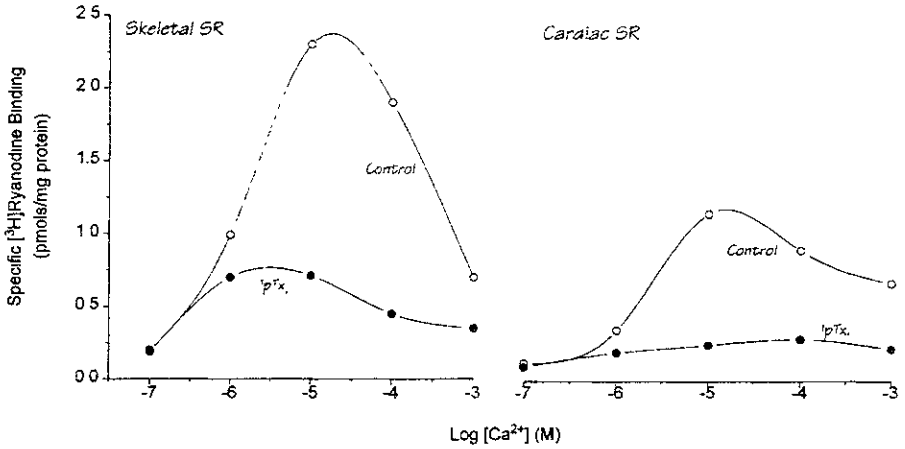

FIG. 6 **Ca^{2+} dependence of $IpTx_i$ inhibition of [^{3}H]ryanodine binding.** Rabbit skeletal (0.3 mg/ml) or pig cardiac (0.4 mg/ml) SR vesicles were incubated for 90 min at 36 °C with 7 nM [^{3}H]ryanodine in the absence (*open circles*) or the presence (*filled circles*) of 200 nM $IpTx_i$. The incubation medium consisted of 0.2 M KCl, 10 mM Na-Hepes (pH 7.2), 1 mM EGTA, and $CaCl_2$ necessary to bring free [Ca^{2+}] to the desired value. The Ca^{2+} EGTA ratios were calculated by a computer program using affinity constants given in Fabiato (33).

cles—The membranes of SR vesicles contain different classes of phospholipids that may serve as substrates for the PLA_2 activity of $IpTx_i$. We reasoned that if the inhibitory properties of $IpTx_i$ were at least partly due to its enzymatic activity, then inhibition should be observed by incubating RyR with supernatant of $IpTx_i$-treated SR vesicles. Control SR vesicles were diluted in binding medium to 1 mg/ml and incubated at 36 °C with 100 nM $IpTx_i$. After 30 min, the $IpTx_i$-treated SR vesicles were pelleted at 32,000 × *g* for 15 min, and a clear supernatant was obtained. The supernatant was then tested for its capacity to inhibit the binding of [^{3}H]ryanodine to cardiac SR vesicles. A control tube that contained only $IpTx_i$ in binding medium without SR vesicles was run in parallel to determine the percentage of inhibition caused by the toxin alone. Fig. 7 shows that increasing concentrations of $IpTx_i$-treated SR supernatant inhibited the binding of [^{3}H]ryanodine dose-dependently (*filled circles*). This inhibition could not be attributed to $IpTx_i$ because the control supernatant reduced binding only marginally (*open circles*). For instance, in the most extreme case, 10 μl of $IpTx_i$-treated SR supernatant inhibited 100% of specific binding; the estimated concentration of $IpTx_i$ in this tube was 10 nM, which inhibited binding only by 22%. Hence, the lipolytic activity of $IpTx_i$ releases from SR vesicles a phospholipid product that is capable of inhibiting RyR activity

Reversibility of $IpTx_i$ Effect—To rule out the possibility that inhibition of RyR by $IpTx_i$ was caused by an irreversible disruption of the channel protein, we incubated SR vesicles (1 mg/ml) with water (control) or with 1 μM $IpTx_i$ ($IpTx_i$-treated SR). After 30 min at 36 °C, an aliquot was taken from each sample. Then, both SR samples were washed twice, pelleted, and prepared for [^{3}H]ryanodine binding. Fig. 8 shows that binding of [^{3}H]ryanodine to $IpTx_i$-treated vesicles was decreased with respect to control after 30 min of incubation. However, these vesicles displayed essentially the same binding as control after washing off $IpTx_i$. Thus, RyRs recover their capacity to bind [^{3}H]ryanodine after treatment with $IpTx_i$. Assuming that the [^{3}H]ryanodine binding assay followed changes in channel gating (8–10, 17, 21), the changes described above suggest that $IpTx_i$ released a phospholipid product that bound to RyRs (or a closely associated regulatory protein) and decreased open probability (p_o); after removal of the phospholipid product, p_o could recover to control levels.

Single Channel Effects of $IpTx_i$—To test directly the above hypothesis and to gain insight on the manner in which $IpTx_i$ inhibits RyRs, we reconstituted swine cardiac RyR in planar lipid bilayers, as described (11, 17) Fig 9 shows traces from continuous recording at −30 mV holding potential in the absence (control) and the presence of increasing concentrations of $IpTx_i$. Channel activity was monitored over 80 s in each condition, and the mean p_o was obtained from current histograms. The traces show that $IpTx_i$ blocks RyR dose-dependently at low concentrations (≈200 nM) $IpTx_i$ decreases the lifetime of the open events, converting long openings into brief, frequently unresolved open events; at higher concentrations, both long and brief openings progressively decrease in frequency until they disappear. A quantitative description of this effect is presented in the open time histograms of Fig. 9*B*. In control, 2,034 events could be fitted with three exponentials with mean open time (τ) = 0.7 ms (66%), 1.9 ms (29%), and 9 8 ms (5%). In the presence of 500 nM $IpTx_i$, the histogram for 801 openings collected during the same period as control was monoexponential

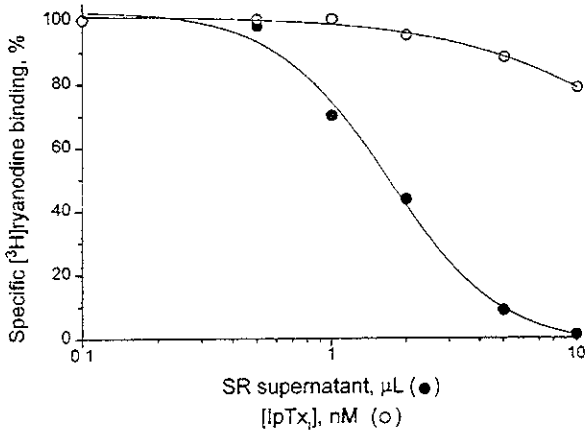

FIG 7 **Inhibition of RyR by supernatant of $IpTx_i$-treated SR.** SR supernatant was prepared as described in the text Binding of [^{3}H]ryanodine to cardiac SR vesicles was carried out in the absence (control, 100%) or presence of indicated volumes of SR supernatant (*filled symbols*). To keep the reaction volume constant, binding medium was added to SR supernatant to a final volume of 10 μl To determine the inhibition caused by $IpTx_i$ alone, control vials containing $IpTx_i$ without SR vesicles were spun in parallel The final concentration of $IpTx_i$ in the binding assays and the inhibition associated with it is shown by the *open symbols*

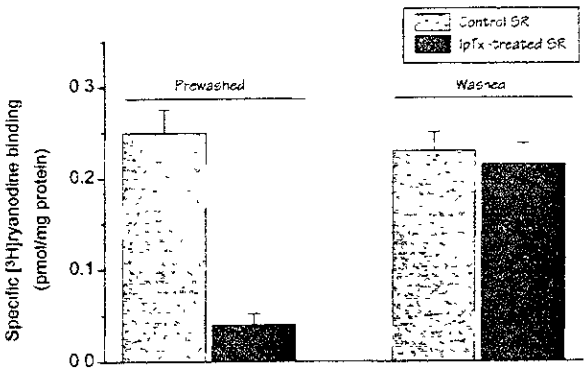

FIG 8 **Reversibility of IpTx$_i$ effect.** Prewashed vesicles are cardiac SR vesicles incubated with water (control) or with 1 μM IpTx$_i$ (IpTx$_i$-treated SR) for 30 min at 36 °C. Washed vesicles are the same control and IpTx$_i$-treated vesicles after being pelleted and washed twice with binding medium. Binding of [^{3}H]ryanodine was conducted in all vesicles and is expressed in absolute values (pmol/mg).

with τ = 0.8 ms. Both the decrease in the frequency and duration of the open events contributed to the decrease in p_o. Fig. 9*C* shows the log dose-response relation of p_o as a function of IpTx$_i$ concentration. The IC_{50} for IpTx$_i$ inhibition was 140 nM. Since this value is in fairly good agreement with that determined in binding experiments (50–80 nM), this indicates that [^{3}H]ryanodine, at the concentrations used, does not interfere with the inhibitory capacity of IpTx$_i$. More importantly, the results show that the lipolytic product of IpTx$_i$ is capable of interacting directly with the RyR or with a closely associated protein that regulates channel gating.

Inhibition of RyR by Fatty Acids—PLA_2 catalyzes the hydrolysis of the S_n2 fatty acyl bond of phospholipids to release free fatty acids and lysophospholipids (22). We tested the effect of each of these lipids separately on RyR activity. Fig. 10*A* shows that lysophosphatidylcholine (lyso-PC), at concentrations up to 300 μM, does not modify substantially the open lifetime or the unitary conductance of cardiac RyR. On the other hand, linoleic acid, an 18-carbon unsaturated fatty acid, blocks RyR at lower concentrations and in a manner that is reminiscent of the blockade produced by IpTx$_i$. Addition of 30 μM linoleic acid to the cytosolic face of the channel decreases the lifetime and the frequency of open events; similar to IpTx$_i$, the fatty acid induces the appearance of openings that are too fast to be resolved by our recording bandwidth.

The potency of several fatty acids to inhibit RyRs was determined by their capacity to inhibit [^{3}H]ryanodine binding to cardiac SR vesicles (Fig. 10*B*). Palmitic acid, a 16-carbon saturated fatty acid, partially inhibited RyR but at concentrations $\geq$100 μM. Arachidonic acid, linoleic acid, and oleic acid, unsaturated fatty acids abundant in SR membranes of cardiac and skeletal muscle (23), totally inhibited RyR with an IC_{50} = 25, 55, and 70 μM, respectively. In agreement with single channel results, lyso-PC and lysophosphatidylethanolamine were unable to modify [^{3}H]ryanodine binding. Thus, not all PLA_2 products are capable of inhibiting RyRs. Among the fatty acids, the potency to inhibit RyRs increases with the carbon chain length and the number of unsaturations.

DISCUSSION

In the present study, we determined the complete amino acid and nucleotide sequence of IpTx$_i$, a heterodimeric protein from the venom of the scorpion *P. imperator*, and unraveled the molecular mechanism by which it inhibits RyRs of cardiac and skeletal muscle.

The deduced amino acid sequence from the cDNA encoding IpTx$_i$ and the amino acid sequence determined by Edman degradation strongly suggests that IpTx$_i$ is synthesized as a precursor of the pre-pro form. Putative signal and connector peptides must be removed to produce mature IpTx$_i$. The proposed signal peptide does have a large content of acidic residues (8 out of the last 18 residues are acidic), a property that is rarely seen in signal peptides. For this reason, we cannot exclude whether the proposed signal peptide codes for another peptide before coding for the large subunit of IpTx$_i$. Regarding the connecting pentapeptide Arg-Arg-Leu-Ala-Arg (positions 105 to 109 in the cDNA sequence of Fig. 3), we gather that several enzymes must be required to eliminate it from the mature protein. Initially, a cleavage must occur at Arg^{109} (monobasic site), as it occurs in the maturation of prosomatostatin and other prohormones (24). Two characteristics distinguish this cleavage site and both are present in the cDNA of IpTx$_i$: (i) Arg (or other basic residue) is immediately preceded by Ala or Leu (or both); (ii) Arg is located three or five residues upstream the basic amino acid involved in the cleavage. Next, another cleavage must occur at Arg^{106} (dibasic site) as is the case during the maturation of diverse peptidic hormones (25). A third enzyme with carboxypeptidase activity must intervene to remove Arg^{105} and Arg^{106}. Because of this complicated pattern of maturation, we assume that the linking of the small and large subunit of IpTx$_i$ must be important for the function of IpTx$_i$.

Mature IpTx$_i$ is composed of a ~3-kDa peptide covalently linked to a ~12-kDa peptide with PLA_2 activity. The large subunit of IpTx$_i$ conserves the most important substructures of secreted PLA_2 from group III (Fig. 4). The His/Asp pair, essential for Ca^{2+} binding, is in position 33/34 of IpTx$_i$ and is preceded in the three PLA_2s by a Cys-Cys-Arg motif; the amino terminus (roughly residues 4–12 of IpTx$_i$), presumably involved in substrate binding, is also highly conserved in the three PLA_2s; lastly, the Cys residues, essential for maintaining proper folding through disulfide bonds, may be used in this group as the elemental frame to align homologous sequences and to identify stretches of sequence that have been inserted or deleted through evolution. Only Cys^{101} of IpTx$_i$ (relative position 104 in Fig. 4) does not match with Cys residues present at the carboxyl terminus of the other PLA_2 shown. It is likely, therefore, that Cys^{101} is involved in the disulfide bridging with Cys^4 of the small subunit.

The presence in IpTx$_i$ of a PLA_2 subunit covalently linked to a small, structurally unrelated peptide is reminiscent of the bipartite arrangement of β-bungarotoxins (26), a group of snake neurotoxins that inhibit neurotransmitter release (27). Each β-bungarotoxin dimer has a PLA_2 subunit covalently bound to a smaller subunit related to Kunitz-type protease inhibitors. Members of the Kunitz superfamily bind to and block voltage-dependent K^+ and Ca^{2+} channels (28). Thus, although the small subunit of IpTx$_i$ showed no homology to members of the Kunitz superfamily, its distinctive arrangement within the toxin suggested that it might act as a blocker. For this reason, it was surprising at first to realize that the small subunit was not directly responsible for inhibiting RyRs. Direct addition of the small subunit to our [^{3}H]ryanodine binding assays failed to inhibit RyRs, as did a synthetic peptide with amino acid sequence similar to that of the small subunit and two additional derivatives designed to avoid interpeptide disulfide bridge formation (Table I). Hence, the target site for the small subunit, or its biological function, remains to be determined.

Given our failure to detect inhibition by the small subunit, we turned our attention to the large subunit of IpTx$_i$. In a reduced and carboxymethylated form, the large subunit of IpTx$_i$ failed to inhibit RyRs (not shown). However, this was not

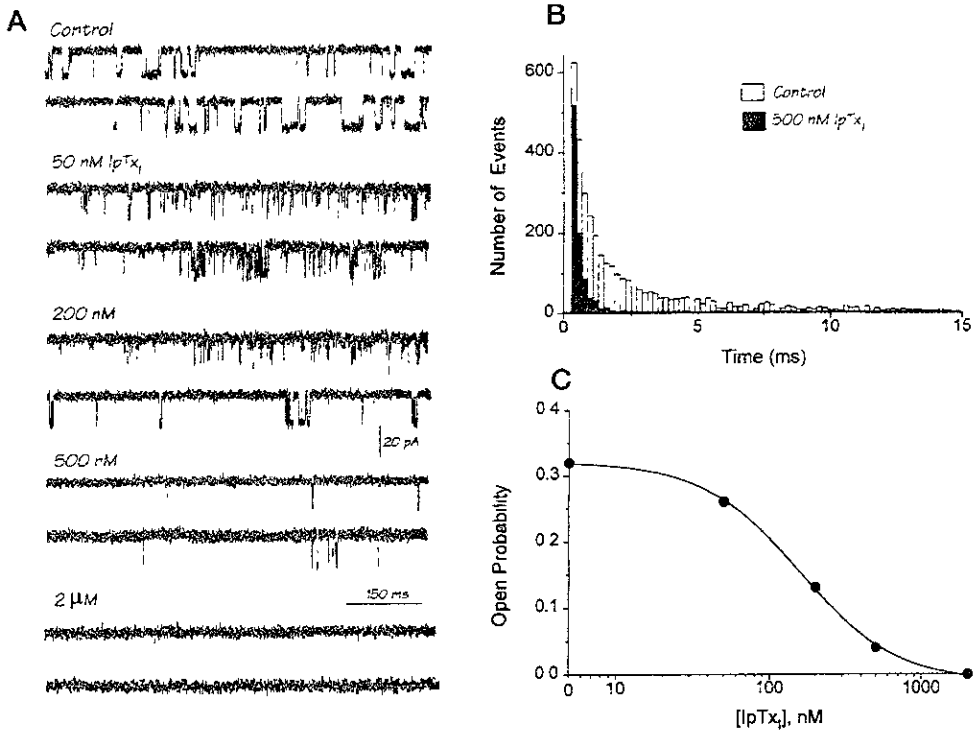

FIG. 9. **Inhibition of cardiac RyR activity by IpTx$_i$.** *A*, pig cardiac RyR were reconstituted in planar lipid bilayers and recorded in the absence (control) and the presence of indicated concentrations of IpTx$_i$. Holding potential: −30 mV. Recording solution: symmetrical 300 mM cesium methane sulfonate and 10 mM Na-Hepes (pH 7.2). Under these conditions, Cs^+ flows from the luminal (trans) to the cytosolic (cis) side of the channel, and openings are represented by downward deflections of the base-line current. *B*, open time histograms in the absence (*open bars*) and the presence (*filled bars*) of 500 nM IpTx$_i$. *C*, plot of open probability (P_o) as a function of [IpTx$_i$]. *Smooth line* is a fit to data points using the expression $p_o = (p_o \text{ control})/1 + ((\text{IpTx}_i)/\text{IC}_{50})^{n_H}$. Where p_o control is the p_o in the absence of IpTx$_i$ (0.32); IC_{50} is the concentration of IpTx$_i$ that produces half-maximal inhibition (140 nM); and n_H is the Hill coefficient (1.22).

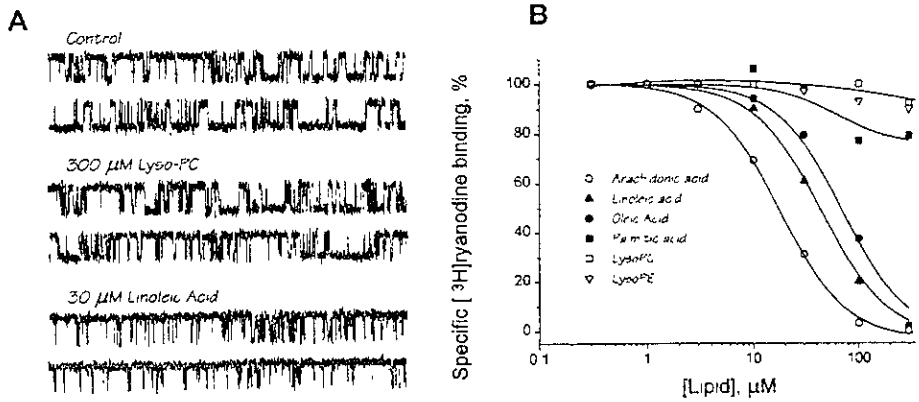

FIG. 10. **Selective inhibition of RyR by fatty acids.** *A*, representative traces of cardiac RyR activity in control (*top trace*) and in the presence of 300 μM lyso-PC (*middle trace*) or 30 μM linoleic acid (*bottom trace*). Openings are represented by downward deflections of the base-line current. For the experiment shown plus two additional experiments, mean p_o was 0.43 ± 0.09 (control), 0.46 ± 0.11 (lyso-PC), and 0.12 ± 0.06 (linoleic acid). *B*, the binding of 7 nM [^{3}H]ryanodine to cardiac SR vesicles was determined in the presence of methanol (maximal concentration = 1%) and this value defined as 100%, or in the presence of indicated concentrations of lipids dissolved in methanol.

surprising given that disulfide bridges are essential to maintain the toxin's three-dimensional structure (20). It was the treatment of IpTx$_i$ with *p*BPB, a covalent modifier of His residues that inhibits PLA_2 activity without affecting disulfide bridges (15), that decreased substantially the capacity of IpTx$_i$ to inhibit RyRs (Fig. 5). This suggested that a lipid product of PLA_2 activity was involved in the inhibition of RyRs. Three separate lines of evidence supported this notion. 1) Inhibition of RyR by IpTx$_i$ was favored by the presence of Ca^{2+} (≥1 μM; Fig. 6), as expected from the Ca^{2+} dependence of enzymatic activity of PLA_2 from group III and several other groups (20). 2) The supernatant of IpTx$_i$-treated SR vesicles could inhibit RyRs even before IpTx$_i$ was present in a concentration large enough to do so (Fig. 7); this indicated that RyR inhibition was brought

about by a lipid product of $IpTx_i$ released from SR vesicles during the incubation period. 3) The kinetics of RyR inhibition by $IpTx_i$ were mimicked by direct addition of linoleic acid (but not of lyso-PC) to the cytoplasmic side of the channel (Figs. 9 and 10); other long chain, unsaturated fatty acids could also inhibit RyRs (Fig. 10). Thus, a potential scenario for inhibition of RyR by $IpTx_i$ is as follows: in the presence of micromolar $[Ca^{2+}]$, the large subunit of $IpTx_i$ catalyzes the hydrolysis of the S_n2 fatty acyl bond of the phospholipids of the SR membrane. The reaction yields free fatty acids and lysophospholipids; the former are released into the incubation medium, and the latter may be liberated or remain embedded into the SR membrane. Free fatty acids bind to RyR or to a closely associated protein that controls gating. At low concentrations, they produce an incomplete block of RyR; higher concentrations gradually block the RyR completely, giving rise to the dose-response relationship of channel activity and $[^3H]$ryanodine binding *versus* $IpTx_i$ concentration of Figs. 1, 5, and 9.

PLA_2s are abundant components of snakes, scorpions, and bee venoms and often constitute the main toxic component to mammals. Although all PLA_2s induce a variety of pathological symptoms including neurotoxicity and myotoxicity (29), they differ in their mechanism of action and in their molecular targets. For example, the snake neurotoxin crotoxin is composed of a PLA_2 subunit and an inhibitory subunit, which keeps the phospholipase inactive until binding to a presynaptic receptor triggers the dissociation of the inhibitory subunit (30). In contrast, notexin from *Netechis scutatus scutatus* is a PLA_2 without an associated subunit that produces muscle paralysis by binding to a specific receptor in the neuromuscular junction (31). $IpTx_i$ is the first example of a scorpion toxin in which a PLA_2 is found chaperoned by a smaller, structurally unrelated subunit. In a previous study (11), we found that several transporters and ion channels of striated muscle including the inositol trisphosphate receptor, the muscarinic receptor, voltage-sensitive Na^+ channels, and the Ca^{2+}-ATPase of SR were insensitive to micromolar concentrations of $IpTx_i$. These results argue against an indiscriminate effect on key molecules of excitation-contraction coupling by the lipolytic action of $IpTx_i$. However, an important question that still remains to be answered concerns the presence of the small subunit of $IpTx_i$. What is its molecular target? What function does it perform to enhance the PLA_2 activity or to suppress it while in transit to the specific receptor? Regardless of the primary site affected, a major contribution of this study was to establish the fact that the molecular mechanism involved in the toxicity of $IpTx_i$ will most likely involve abnormalities in intracellular Ca^{2+} mobilization due to blockade of RyRs.

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The Mechanism of Inhibition of Ryanodine Receptor Channels by Imperatoxin I, a Heterodimeric Protein from the Scorpion *Pandinus imperator**

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We present an in-depth analysis of the structural and functional properties of Imperatoxin I ($IpTx_i$), an ~15-kDa protein from the venom of the scorpion *Pandinus imperator* that inhibits Ca^{2+} release channel/ryanodine receptor (RyR) activity (Valdivia, H. H., Kirby, M. S., Lederer, W. J., and Coronado, R. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 12185–12189). A cDNA library was prepared from the venomous glands of this scorpion and used to clone the gene encoding $IpTx_i$. From a single continuous messenger RNA, the information coding for the toxin is translated into two mature polypeptide subunits after elimination of a basic pentapeptide. The $IpTx_i$ dimer consists of a large subunit (104-amino acid residues) with phospholipase A_2 (PLA_2) activity covalently linked by a disulfide bond to a smaller (27 amino acid residues), structurally unrelated subunit. Thus, $IpTx_i$ is a heterodimeric protein with lipolytic action, a property that is only shared with β-bungarotoxins, a group of neurotoxins from snake venoms. The enzymatic subunit of $IpTx_i$ is highly homologous to PLA_2 from bee (*Apis mellifera*) and lizard (*Heloderma horridum*) venoms. The small subunit has no significant similarity to any other known peptide, including members of the Kunitz protease inhibitors superfamily that target the lipolytic effect of β-bungarotoxins. A synthetic peptide with amino acid sequence identical to that of the small subunit failed to inhibit RyR. On the other hand, treatment of $IpTx_i$ with *p*-bromophenacylbromide, a specific inhibitor of PLA_2 activity, greatly reduced the capacity of $IpTx_i$ to inhibit RyRs. These results suggested that a lipid product of PLA_2 activity, more than a direct $IpTx_i$-RyR interaction, was responsible for RyR inhibition.

Scorpion venoms contain families of small basic proteins that modify the gating mechanism of Na^+ channels or block with high affinity K^+ channels of excitable cells (1, 2). These toxins have been invaluable tools in the identification, purification, structural mapping, and functional characterization of the corresponding ionic channels. The study of other ionic channels have also been aided by toxins from poisonous animals. For instance, snake venoms contain potent neurotoxins that block acetylcholine receptors (3), and snail and spider venoms contain small molecular weight proteins directed against neuronal Ca^{2+} channels (4, 5). A Cl^- channel-specific blocker peptide was also recently isolated from a scorpion venom (6). Thus, there exist a vast array of natural ligands useful for structural and functional characterization of ionic channels.

The Ca^{2+} release channel of SR[1] constitutes the major pathway for Ca^{2+} release during the process of excitation-contraction coupling in cardiac and skeletal muscle (7). The Ca^{2+} release channel binds the plant alkaloid ryanodine with nanomolar affinity, hence the name ryanodine receptor (RyR). Ryanodine has been an invaluable tool in the structural and functional characterization of RyR. The alkaloid binds to a conformationally sensitive domain on the RyR protein and may be used in binding assays as an index of the functional state of the channel (8, 9). However, ryanodine displays extremely slow dissociation kinetics that make its effect practically irreversible. Furthermore, certain concentrations of ryanodine may open RyRs while others may block them (10), leading to ambiguous results.

From the venom of the African scorpion *Pandinus imperator*, we isolated Imperatoxin I ($IpTx_i$), a ~15-kDa protein that inhibited [^{3}H]ryanodine binding to cardiac and skeletal SR by blocking RyR channels (11). At concentrations well above the half-maximal effective concentrations (ED_{50}) exhibited for RyR, $IpTx_i$ did not modify the binding of ligands targeted against other transporters and ionic channels of striated muscle (11). $IpTx_i$ blocked RyR rapidly and reversibly, and when injected in ventricular cells it decreased twitch amplitude and intracellular Ca^{2+} transients, suggesting a selective blockade of Ca^{2+} release from the SR (11).

In this study, we carried out an in-depth analysis of the mechanism of action of $IpTx_i$ on RyRs of cardiac and skeletal muscle. We determined the complete amino acid and nucleotide sequence of $IpTx_i$ and show that, like β-bungarotoxins, $IpTx_i$ is a heterodimeric protein composed of a high molecular weight subunit with PLA_2 activity and a small, structurally unrelated

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; *p*BPB, *p*-bromophenacyl bromide; PLA_2, phospholipase A_2; RyR, ryanodine receptor; lyso-PC, lysophosphatidylcholine; HPLC, high performance liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.

subunit. We also show that free fatty acids, lipid products of $IpTx_i$-PLA_2 activity, are involved in the inhibition of RyR. $IpTx_i$ thus offers an alternative way to block RyR distinct from ryanodine and other ligands that require a physical interaction with the RyR protein.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Protease lysine C (Lys-C) and restriction enzymes were from Boehringer Mannheim. Chemicals and solvents for peptide sequencing were from Millipore Co. Primers for polymerase chain reaction (lambda gt11 forward and reverse; 1218 and 1222) and Vent^R polymerase were from New England BioLabs. DNA sequencing kit (Sequenase version II), was from U. S. Biochemical Corp. Subcloning and sequencing vector (pBluescript phagemid; pKS), M13 −20, and M13 reverse sequencing primers were from Stratagene. Brain phosphatidylethanolamine and brain phosphatidylserine were from Avanti Polar Lipids. [^{3}H]Ryanodine was from DuPont NEN. *p*-Bromophenacyl bromide (*p*BPB) was from Sigma.

Purification of $IpTx_i$—P. imperator venom was obtained by electric stimulation of scorpions maintained alive in the laboratory. Venom (120 mg per batch) was suspended in double distilled water and centrifuged at 15,000 × *g* for 30 min. The supernatant was applied onto a column (0.9 × 190 cm) of Sephadex G-50 superfine (Pharmacia Biotech Inc.). Fractions were eluted with 20 mM NH_3OAc (pH 4.7) at a flow rate of 20 ml/h. Fraction II containing $IpTx_i$ was applied to a column (0.9 × 30 cm) of carboxymethyl (CM)-cellulose 32 (Whatman) equilibrated with 20 mM NH_3OAc (pH 4.7). Peptides were eluted at a flow rate of 20 ml/h with a linear gradient of 250 ml of 20 mM NH_3OAc (pH 4.7) and 250 ml of the same buffer containing 0.55 M NaCl. Peptides displaying capacity to inhibit [^{3}H]ryanodine binding and phospholipase activity were dialyzed against deionized water (3 × 30 min), concentrated by lyophilization, and injected into a C_4 reverse-phase HPLC column (Vydac). $IpTx_i$ was eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid running at 1 ml/min for 60 min. The purity and identity of $IpTx_i$ was confirmed by amino acid sequence analysis, as described for Na^+ channel-blocking peptides (12). $IpTx_i$ was quantified either by amino acid analysis or by absorbance at 280 nm ($A_{280\ nm}$) using an extinction coefficient (ϵ) = 14,952.

Amino Acid Analysis and Microsequencing of $IpTx_i$—Amino acid analysis was performed on samples hydrolyzed in 6 N HCl with 0.5% phenol at 110 °C in evacuated, sealed tubes as described (13). Reduction of $IpTx_i$ with dithiothreitol and alkylation with iodoacetic acid was performed as described (13). Reduced and alkylated $IpTx_i$ was cleaved with protease Lys-C in 200 μl of 25 mM Tris-HCl (pH 7.2), and 1.0 mM EDTA, at a enzyme:peptide ratio = 1:100. Microsequence determination of native, reduced, and carboxymethylated toxin, and their peptide fragments was carried out with a 6400/6600 MilliGen/Biosearch Prosequencer, using the peptide adsorbed protocol on CD Immobilon membranes.

Cloning and Sequencing of the cDNA Encoding $IpTx_i$—Two oligonucleotides encoding for two different regions of $IpTx_i$ were synthesized as described (13). Oligonucleotide 1 (5′-AC(*N*)ATGTGGGG(*N*)AC(*N*)AA(G/A)TGGTG-3′; where *N* means any nucleotide) encoded for the first 8 amino acids of the amino terminus of the large subunit of $IpTx_i$. Oligonucleotide 2 (5′-GA(G/A)GC(*N*)GG(*N*)TA(T/C)GG(*N*)GC(*N*)TGGGC-3′) encoded for residues 14–21 of the small subunit. Total RNA was purified from the venomous glands (telsons) of 30 scorpions. The telsons were pulverized in liquid nitrogen and poured into 10 ml of GT buffer (4 M guanidinium isothiocyanate, 0.025 M sodium citrate (pH 7.0), 0.5% *N*-lauryl sarcosine, and 0.03 M β-mercaptoethanol), vortexed, and centrifuged at 5,000 × *g* for 10 min. One ml of 2 M sodium acetate (pH 4) was added to the recovered supernatant. The resulting solution was extracted with 10 ml of water-saturated phenol plus 2 ml of chloroform: isoamyl alcohol (49:1, v:v), allowing to sit on ice for 15 min. After centrifuging at 10,000 × *g* for 20 min at 4 °C, the aqueous layer was precipitated with 1 volume of isopropyl alcohol, incubated at −20 °C for 1 h, and centrifuged at 10,000 × *g* for 20 min at 4 °C. The pellet was dissolved in 0.3 ml of GT buffer and 0.3 ml of isopropyl alcohol, incubated at −20 °C for 1 h, and pelleted again. The pellet was washed with 95% ethanol, briefly dried under vacuum, and resuspended in RNase-free water. Messenger RNA was purified following the instructions of the Hybond-mAP protocol (messenger affinity paper; Amersham Corp., RPN.1511). cDNA synthesis was performed as described (13) from 5 μg of mRNA. The cloning of the cDNA library was also performed as described (13). The screening of the cDNA library was performed with oligonucleotides 1 and 2 separately, but the clones detected with oligonucleotide 1 were analyzed first. Inserts of cDNA from positive clones were polymerase chain reaction-amplified using λ gt11 forward and reverse primers. Polymerase chain reaction products were purified from agarose gel and ligated into the *Eco*RV site of pBluescript (pKS) phagemid. The ligation reactions were used to transform *Escherichia coli* DH5-α cells. Plasmid DNA from white colonies was digested with *Bam*HI and *Hin*dIII to verify the size of the original inserts. Clones of interest were sequenced using the Sequenase^R kit version 2 (U. S. Biochemical Corp.) on both strands. Oligonucleotides λ gt11 forward, λ gt11 reverse, M13 -20, and M13 reverse were used for sequencing.

Sequence Comparisons—The amino acid sequence of the large and small subunits of $IpTx_i$ were compared with those of other proteins deposited in the protein data base of GenBank (Los Alamos National Laboratory, Los Alamos, NM) by computer analysis using the program Blitz version 1.5 (Biocomputing Research Unit, University of Edinburgh, UK).

Determination of Phospholipase A_2 Activity—Phospholipase A_2 activity was determined by the titration method of Shiloah *et al.* (14), using dilute egg yolk (1:20 in saline solution) as substrate. Liberation of acid was measured at pH 8.0 and 37 °C by titration with 5 mM NaOH under a constant stream of N_2. One unit of enzyme is defined as the amount of enzyme that liberates 1 μmol of free fatty acid/min under the above conditions. Inhibition of phospholipase A_2 with *p*BPB was carried out as described by Díaz *et al.* (15). One hundred μl of 200 μM $IpTx_i$ in 35 mM Tris (pH 8.0) were mixed with 10 μl of 2 mM *p*-BPB in acetone and incubated for 16 h at room temperature under dim light. The reaction was stopped by filtration in a Sephadex G-10 column. *p*BPB-treated $IpTx_i$ elutes in the void volume of the column, whereas the excess of *p*BPB is retarded.

Synthesis of the Small Subunit of $IpTx_i$—The small subunit of $IpTx_i$ was synthesized by the solid phase method of Merrifield (16), using *t*-butyloxycarbonyl-amino acids. This subunit contains a Cys residue at position 4. To avoid formation of inter-peptide disulfide bonds, Cys[4] was substituted by Ala (peptide A), Met (peptide M), or Cys (peptide C) protected with 3-nitro-2-pyridinesulfonyl (thiol-protecting group that will not allow formation of disulfide bonds). At the end of the synthesis all three peptides were separated in a C_{18} reverse-phase HPLC column, using the conditions described above. The purity of the synthetic peptides was confirmed by both amino acid analysis and microsequencing, as described above.

[^{3}H]Ryanodine Binding Assays—[^{3}H]Ryanodine binding to pig cardiac and rabbit skeletal SR vesicles was carried out for 90 min at 36 °C in 0.1 ml of 0.2 M KCl, 1 mM Na_2EGTA, 0.995 mM $CaCl_2$, 10 mM Na-Pipes (pH 7.2). The calculated free Ca^{2+} was 10 μM. [^{3}H]Ryanodine (68.4 Ci/mmol) was diluted directly in the incubation medium to a final concentration of 7 nM. Protein concentration was 0.2–0.4 and 0.3–0.5 mg/ml for skeletal and cardiac SR, respectively. Samples were filtered on Whatman GF/B glass fiber filters and washed twice with 5 ml of distilled water. A Brandel M24R cell harvester was used for filtration. Nonspecific binding was determined in the presence of 10 μM unlabeled ryanodine and has been subtracted from each sample.

Planar Bilayer Technique—Recording of single RyR in lipid bilayers was performed as described previously (17). Briefly, a phospholipid bilayer of phosphatidylethanolamine:phosphatidylserine (1:1 dissolved in *n*-decane to 20 mg/ml) was formed across an aperture of ~300 μm diameter in a delrin cup. The cis chamber (900 μl) was the voltage control side connected to the head stage of a 200 A Axopatch amplifier, and the trans chamber (800 μl) was held at virtual ground. Both chambers were initially filled with 50 mM cesium methane sulfonate and 10 mM Tris/Hepes (pH 7.2). After bilayer formation, cesium methane sulfonate was raised to 300 mM in the cis side, and 100–200 μg of SR vesicles were added. After detection of channel openings, Cs^+ in the trans chamber was raised to 300 mM to collapse the chemical gradient. Single channel data were collected at steady voltages (−30 mV) for 2–5 min. Channel activity was recorded with a 16-bit VCR-based acquisition and storage system at a 10-kHz sampling rate. Signals were analyzed after filtering with an 8-pole Bessel filter at a sampling frequency of 1.5–2 kHz. Data acquisition and analysis were done with Axon Instruments software and hardware (pClamp v6.0, Digidata 200 AD/DA interface).

RESULTS

Purification of $IpTx_i$—Purification of $IpTx_i$ from *P. imperator* venom was performed in three chromatographic steps as described under "Experimental Procedures" and shown in Fig. 1. After fractionation of whole venom in Sephadex G-50 (Fig. 1*A*), five fractions were collected and assayed for effects on [^{3}H]ryanodine binding. Fraction 2 contained polypeptides in the

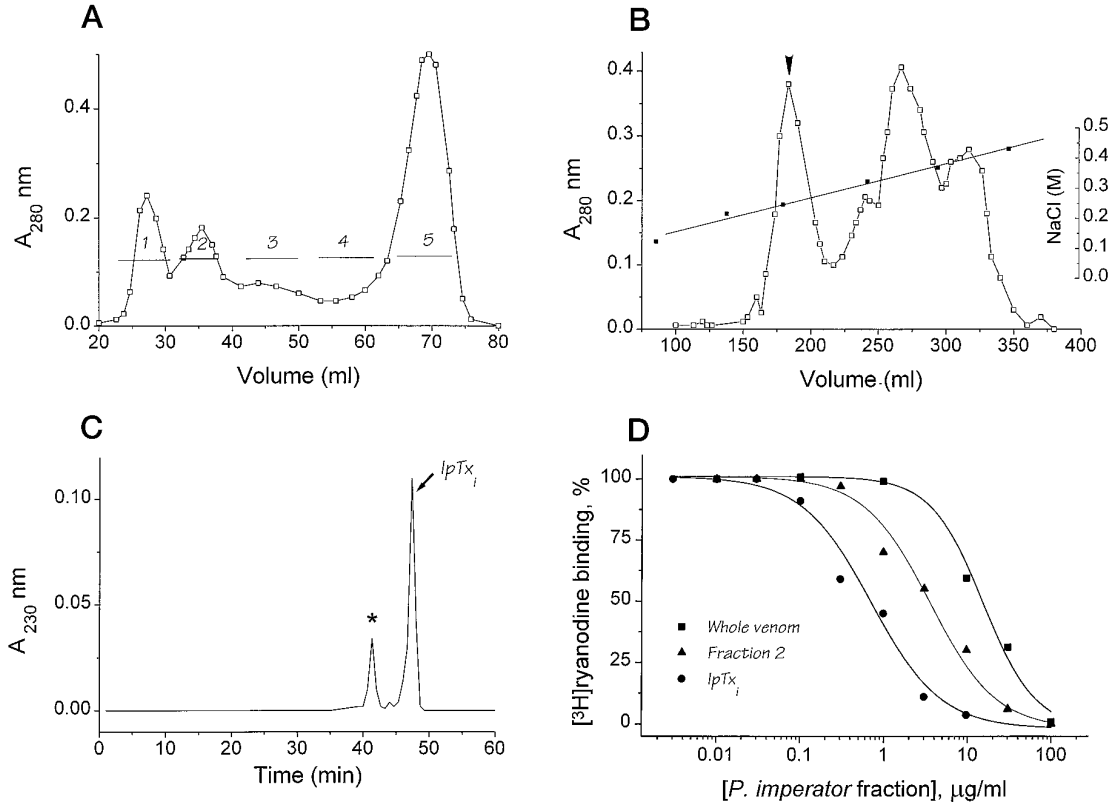


FIG. 1. **Purification of IpTx$_i$.** *A*, *P. imperator* soluble venom (120 mg of protein) was applied to a Sephadex G-50 column (0.9 × 190 cm) equilibrated and run with 20 mM ammonium acetate buffer (pH 4.7). 5-ml samples were collected and tested for their capacity to inhibit [^{3}H]ryanodine binding. Only fraction 2 totally inhibited [^{3}H]ryanodine binding. *B*, fraction 2 was further separated through a CM-cellulose column (0.9 × 30 cm), equilibrated, and run with 20 mM ammonium acetate buffer (pH 4.7). A linear gradient of sodium chloride resolved 5 subfractions, the first of which (indicated by the *arrow*) contained IpTx$_i$. *C*, the subfraction from the CM-cellulose column containing IpTx$_i$ was lyophilized and injected into a C_4 reverse-phase HPLC column and eluted with a 0–100% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The peak labeled with an *asterisk* was not studied further. *D*, dose-response curve for whole venom and purified components. [^{3}H]Ryanodine (7 nM) was incubated with cardiac SR protein in 0.2 M KCl, 10 μM $CaCl_2$, 10 mM Na-Hepes (pH 7.2) in the absence (control, 100%) and the presence of indicated concentrations of venom components. Nonspecific binding was determined in the presence of 20 μM ryanodine and has been subtracted from this and subsequent results.

range of 8–16 kDa and was the only fraction that inhibited [^{3}H]ryanodine binding. The second step in the purification of IpTx$_i$ consisted of ion-exchange chromatography in CM-cellulose (Fig. 1*B*). The fraction containing IpTx$_i$ (indicated by the *arrow*) eluted early in the run, when the concentration of NaCl was ~160 mM. Fig. 1*C* shows the chromatographic profile of IpTx$_i$ after elution from a reverse-phase C_4 HPLC column. Only a minor contaminant was present (labeled with *asterisk*), which was discarded for further studies of IpTx$_i$ structure. However, amino acid sequence analysis of purified IpTx$_i$ yielded two different amino acids per reaction cycle, with an apparent equivalent stoichiometry. This indicated either that a contaminant was still present at the end of our purification procedure or that IpTx$_i$ was composed of two different peptide subunits.

We determined the dose-response relationship for each of the active venom components to assess their potency to inhibit RyRs (Fig. 1*D*). Whole *P. imperator* venom and fraction 2 inhibited [^{3}H]ryanodine binding to *cardiac* SR with a concentration of 20.2 and 4.3 μg/ml, respectively, yielding the half-maximal effect (IC_{50}); IC_{50} for pure IpTx$_i$ was 0.7 μg/ml. Based on this value and the molecular mass of IpTx$_i$ (~15 kDa), the estimated apparent dissociation constant (K_d) was 46 nM. Essentially identical results were obtained when *skeletal* SR was used for displacement studies (not shown).

Electrophoretic Analysis of IpTx$_i$—To elucidate whether IpTx$_i$ was composed of two subunits, an aliquot of IpTx$_i$ was reduced and alkylated. The modified toxin was then chromatographed in a Bio-Gel P30 column (Fig. 2*A*) to eliminate reaction by-products and excess of reagents. Three peaks were obtained (Fig. 2*A*) and analyzed by SDS-PAGE (Fig. 2*B*). Peak 1 (labeled *Red*, abbreviation for reduced) had lower molecular mass than native IpTx$_i$ (labeled *Nat*); peak 2 could not be resolved in the same gel (not shown), but direct sequencing indicated that it corresponds to a ~3-kDa peptide (Fig. 3*A*); peak 3 was not peptidic in nature, indicating that it corresponded to the reducing and alkylating reagents. Fig. 2*C* shows that the apparent molecular mass of native IpTx$_i$ was ~15 kDa and that of the reduced IpTx$_i$ was ~12 kDa. Thus, treatment of IpTx$_i$ with thiol-reducing agents separates a large (~12 kDa) from a small (~3 kDa) subunit.

Peptide Sequence Determination—The reduced and alkylated derivatives of IpTx$_i$ (peak 1 and 2 of Fig. 2*A*) were sequenced by direct Edman degradation. As shown in Fig. 3*A*, we identified the first 27 amino acid residues of the large subunit, and all 28 amino acid residues of the small subunit. To identify the remaining residues, the large subunit was cleaved with Lys-C endopeptidase, which yielded three peptide fragments that eluted with different retention times in an HPLC column (data not shown). The amino acid sequence of each of these peptides, obtained also by Edman degradation, is *underlined* in Fig. 3*A*.

Cloning and Sequencing of the cDNA Encoding IpTx$_i$—Two oligodeoxynucleotides synthesized according to the amino acid sequence of stretch 1–8 of the large subunit and stretch 14–21 of the small subunit were used to screen a cDNA library, constructed from the venomous glands of *P. imperator* scorpi-

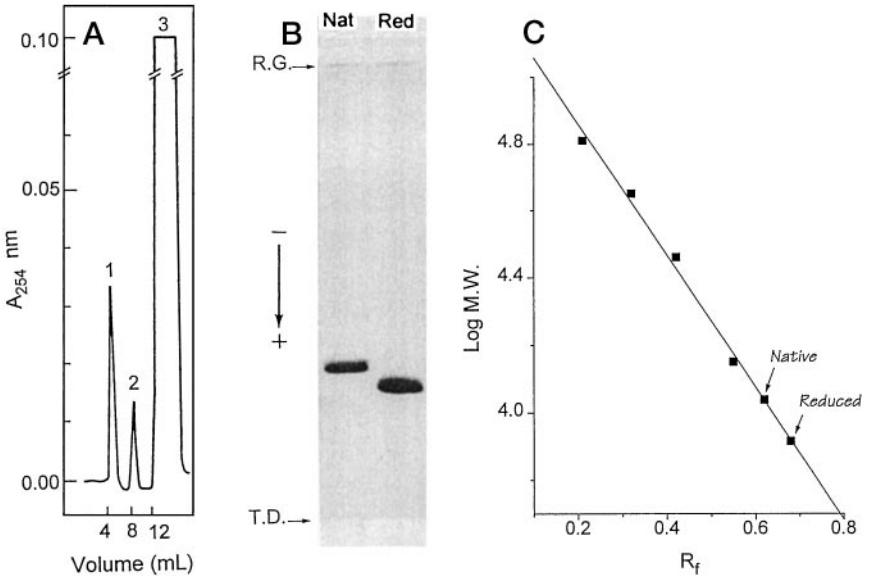
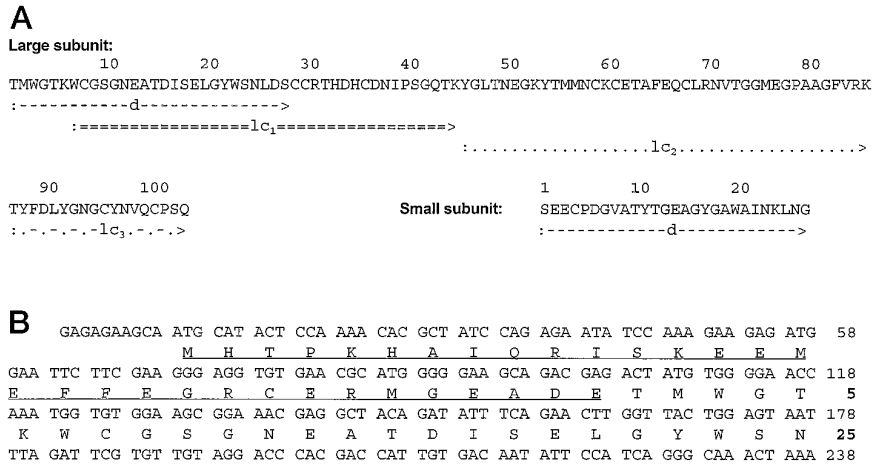


FIG. 2. **Separation of large and small subunits of IpTx$_i$.** *A*, IpTx$_i$ (100 μg) was reduced and alkylated as described under "Experimental Procedures," applied to a Bio-Gel P30 column (0.9 × 27 cm), and eluted with 10% acetic acid. Peak 1 corresponds to a ~12-kDa peptide, peak 2 to a ~3-kDa peptide (determined by direct sequencing), and peak 3 to reaction by-products. *B*, SDS-PAGE analysis of 3 μg of native IpTx$_i$ (*Nat*) and 3 μg of peak 1 (*Red*). Gel was 10% polyacrylamide. Proteins were stained with Coomassie Blue. The running gel (*R.G.*) and the tracking dye (*T.D.*) positions are indicated by *arrows*. *C*, the retention factor (R_f) for various molecular weight markers is plotted as a function of $\log_{10}$ molecular weight (*M.W.*). The apparent molecular weight for native and reduced IpTx$_i$ was extrapolated from a linear regression to the data points.



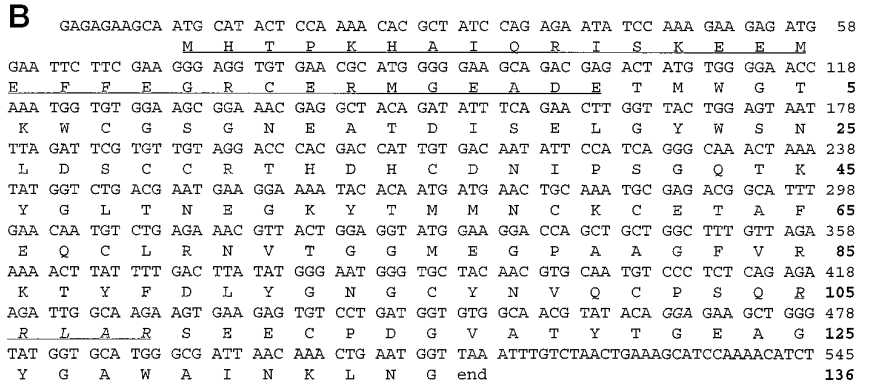


FIG. 3. **Amino acid and nucleotide sequences of IpTx$_i$.** *A*, the complete amino acid sequence of IpTx$_i$ was obtained from direct Edman degradation using peak 1 (large subunit) and peak 2 (small subunit) of Fig. 2*A* (labeled *d*) and from proteolytic fragments (lc_1, lc_2, and lc_3) of peak 1. To obtain the proteolytic fragments, peak 1 (100 μg) was incubated for 4 min at 36 °C with 1 μg of Lys-C endopeptidase in Tris buffer as specified in the Boehringer catalog. The reaction products were separated by HPLC, essentially as described in the legend to Fig. 1*A*. *Numbers on top* of the amino acid sequence correspond to the amino acid positions in the primary structure. *B*, nucleotide sequence of the cDNA gene encoding for the entire IpTx$_i$ molecule with the deduced amino acid sequence below each base triplet. The putative signal peptide sequence (relative position −31 to −1) is *underlined*. The pentapeptide *RRLAR* (relative position 105–109) is not found in the mature protein and is assumed to be cleaved during processing. *Numbers on right* refers to the nucleotide and amino acid positions.

ons. These oligodeoxynucleotides were used as probes to isolate a full-length IpTx$_i$ cDNA clone. Both probes led to the isolation of the same cDNA clone. Its complete nucleotide sequence is shown in Fig. 3*B*. This sequence contained an open reading frame of 167 amino acids encompassing 1) a putative signal peptide (first underlined 31 amino acids, positions −31 to −1); 2) the mature ~12-kDa, large subunit of IpTx$_i$ (104 amino acids, positions 1–104); 3) a putative connector pentapeptide (*RRLAR,* positions 105–109); and 4) the mature small subunit (27 amino acids; positions 110–136). Thus, a single continuous cDNA clone encoded the two polypeptide subunits of IpTx$_i$. This finding confirmed that IpTx$_i$ is a heterodimeric protein. Since treatment of IpTx$_i$ with thiol-reducing agents (Fig. 2*A*) effectively separates both subunits, this suggests that they are covalently linked by a disulfide bridge.

Sequence Homology of IpTx$_i$ Subunits—A comparison of the amino acid sequence of the two subunits of IpTx$_i$ with sequences available from GenBank revealed intriguing results. First, the small subunit shared no significant similarity with sequences of scorpion peptide blockers of Na^+, K^+, or Cl^- channels, with members of the Kunitz protease inhibitor superfamily, or with any other sequence deposited in the protein data base. Thus, this small subunit constitutes a new class of scorpion peptides. On the other hand, Fig. 4 shows that the large subunit of IpTx$_i$ was 38% homologous to PLA_2 from honey bee (*A. mellifera*) venom and 35% homologous to PLA_2 from heloderma (*H. horridum*) venom. These two PLA_2s compose group III of secreted PLA_2 (18), whose main features are low molecular mass (~14 kDa), high disulfide bond content, and strict dependence on Ca^{2+} for lipolytic effect. Since the major criterion for classification is sequence homology more than function, IpTx$_i$ may be classified within this group with the remarkable difference, however, of possessing an accessory protein.

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-3  1                                                    50
    TMWGTKWCGSGNEATDISELGYWSNLDSCCRTHDHC-DNIPS-GQTKYGL
  IIYPGTLWCGHGNKSSGPNELGRFKHTDACCRTHDMCPDVM-SAGESKHGL
 GAFIMPGTLWCGAGNAASDYSQLGTEKDTDMCCRDHDHC-ENWISALEYKHGM

    51                                                  100
    TNEGKYTMMNCKCETAFEQCLRNVTGGMEGPAAGFVRKTYFD-LYGNGCY
    TNTASHTRLSCDCDDKFYDCLKNSADTISSY---FVGKMYFN-LIDTKCY
    RNYYPSTISHCDCDNQFRSCLMKLKDGTADY----VGQTYFNVLKIP-CF

    101                                                 150
    NVQCPSQ ....................................
    KLEHPVTGCGERTEGR-CLHYTVDKSKPK-VYQWFDLRKYXXNSRG-GSX
    ELEEGE-GCVDWNFWLECTESKIMPVAKLVSAAPYQAQAETQSGEG ...

    151
    ........ (IpTx_I)
    EYHFEIVY (Bee phospholipase)
    ........ (Heloderma phospholipase)
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FIG. 4. **Amino acid sequence homology between the large subunit of $IpTx_i$ and PLA_2s from group III.** The amino acid sequence of the large subunit of $IpTx_i$ (*first row*) was aligned with that of bee (*A. mellifera*, *second row*) and lizard (*H. suspectum*, *third row*) venom PLA_2. A few gaps (*dashes*) were introduced to enhance similarities. *Bold letters* indicate exact homology for the three PLA_2s. *Dots* indicate end of the sequence, whereas *X* indicates unknown residues, as indicated in the reference source. Amino acid sequences taken from $IpTx_i$, this work; bee PLA_2, GeneBank Accession No. X16709; *Heloderma suspectum* PLA_2, Gomez *et al.* (32).

Lack of Inhibition by the Short Subunit of $IpTx_i$—We tested if the small subunit of $IpTx_i$ was responsible for blocking RyR. Reduced and carboxymethylated small subunit (peak 2 of Fig. 2*A*), at concentrations up to 15 μM, had no effect on the binding of [^{3}H]ryanodine to cardiac or skeletal RyR (data not shown). To discard the presence of spurious reagents as being the cause of negative results, we synthesized the small subunit in three distinctive forms as follows: peptide C was similar to the native small subunit, and peptides A and M were also similar to the native small subunit, except that Cys^4 was substituted by Ala and Met, respectively. This avoided the formation of interpeptide disulfide bridges caused by oxidation of Cys^4. The three peptides were HPLC-purified before testing. Table I shows that none of the purified synthetic peptides had significant effect on the binding of [^{3}H]ryanodine to cardiac RyR or on the binding of [^{3}H]PN200–110 to voltage-dependent Ca^{2+} channels of sarcolemma (19).

The Phospholipase A_2 Activity of $IpTx_i$ Is Important to Inhibit RyR—We next investigated if the large subunit of $IpTx_i$, or its enzymatic activity, was involved in RyR inhibition. The reduced and carboxymethylated form of the large subunit of $IpTx_i$ (peak 1) was incapable of inhibiting [^{3}H]ryanodine binding to cardiac or skeletal RyR. However, this was expected given that internal disulfide bridges are essential for preservation of the toxin's tridimensional structure as well as for expression of its enzymatic activity (20). We thus resorted to *p*BPB, a covalent modifier of His residues that specifically blocks phospholipase A_2 activity (15) without affecting disulfide bonds. The rate of fatty acid liberation by 1 mol of $IpTx_i$ was 384 ± 26 and 41 ± 12 mol min^{-1} in control and after incubation with *p*BPB, respectively (n = 4; data not shown). Thus, treatment of $IpTx_i$ with *p*BPB decreased substantially its PLA_2 activity. Fig. 5 shows that *p*BPB-treated $IpTx_i$ decreased dramatically its capacity to inhibit the binding of [^{3}H]ryanodine to cardiac and skeletal RyR (*open symbols*). This was in contrast to control $IpTx_i$ (a batch of $IpTx_i$ that underwent the same treatment as *p*BPB-treated $IpTx_i$, except that *p*BPB was omitted), which retained its capacity to inhibit cardiac and skeletal RyR with high affinity (*filled symbols*).

Ca^{2+} Dependence of $IpTx_i$ Effect—The enzymatic activity of PLA_2 from group III requires Ca^{2+} for catalysis (20). Therefore, we expected that if the inhibition of RyRs by $IpTx_i$ resided in its enzymatic activity, inhibition should be apparent only in the presence of micromolar [Ca^{2+}]. Fig. 6 shows that Ca^{2+} was essential for binding of [^{3}H]ryanodine to RyRs and for detection of the $IpTx_i$ effect. The *left panel* shows the Ca^{2+} dependence of [^{3}H]ryanodine binding to *skeletal* SR and the effect of $IpTx_i$. Specific binding in control (*open circles*) had a threshold for detection at 100 nM [Ca^{2+}] (*p*Ca 7) and was optimal at 10–100 μM [Ca^{2+}]. Higher [Ca^{2+}] inhibited binding, giving rise to a *bell-shaped curve*. In the presence of $IpTx_i$ (*filled circles*), the binding curve was dramatically decreased in absolute values. The percentage of $IpTx_i$ inhibition was 5.5, 32, and 71% at *p*Ca 7, 6, and 5, respectively. No further inhibition was observed at higher [Ca^{2+}]. Thus, the degree of $IpTx_i$ inhibition increased with [Ca^{2+}].

In *cardiac* SR (Fig. 6, *right panel*), the Ca^{2+} dependence of [^{3}H]ryanodine binding also had a threshold for detection at *p*Ca 7 and a maximum at *p*Ca 5 (control, *open circles*). However, unlike skeletal SR, inactivation of binding by high [Ca^{2+}] was not pronounced. Binding decreased only by 30% with respect to maximum in cardiac RyRs, and it decreased by 80% in skeletal RyRs. This distinctive response of RyR to Ca^{2+} is also displayed by individual RyR reconstituted in lipid bilayers (8, 21), indicating that the binding assay effectively tracks the activity of the receptor. In the presence of $IpTx_i$, the binding curve was markedly decreased. The percentage of inhibition was 10, 38, and 83% at *p*Ca 7, 6, and 5, respectively. Thus, although cardiac and skeletal RyRs respond differently to Ca^{2+}, the inhibitory effect of $IpTx_i$ increased equally with [Ca^{2+}] in both isoforms.

Inhibition of RyR by Supernatant of $IpTx_i$-treated SR Vesi-

TABLE I

Effect of synthetic peptide analogs of the small subunit of $ipTx_i$ on the binding of [^{3}H]ryanodine and [^{3}H]PN200–110 to cardiac sarcolemma and SR vesicles

Peptide concentration tested was 10 μM. Results are the mean ± S.D. of three independent determinations.

	[^{3}H]Ryanodine binding[a]	[^{3}H]PN200–110 binding[b]
	%	%
Control	100	100
Peptide A	116 ± 8	100 ± 6
Peptide M	110 ± 4	111 ± 12
Peptide C	106 ± 5	111 ± 7

[a] Binding of [^{3}H]ryanodine to SR vesicles was carried out as described in the text.

[b] Binding of [^{3}H]PN200–110 to cardiac sarcolemma was performed as described (19).

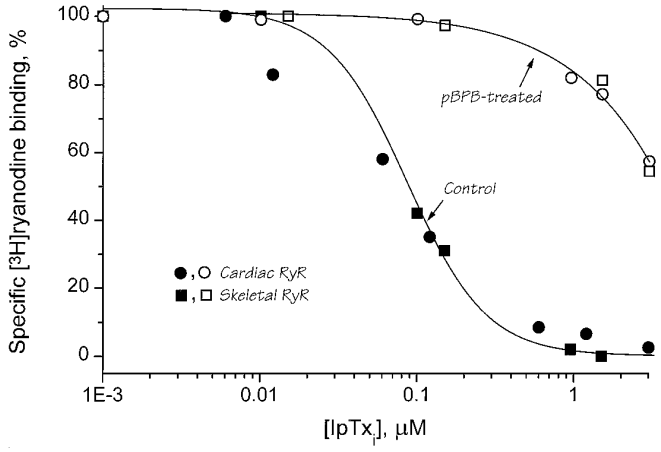

FIG. 5. **Treatment with a PLA_2 inhibitor lowers the potency of $IpTx_i$ to inhibit RyR.** $IpTx_i$ (200 μM) was incubated with 2 mM *p*BPB (*filled symbols*) for 16 h as described under "Experimental Procedures" or with acetone (*open symbols*), the drug vehicle, as control. Binding of [^{3}H]ryanodine to cardiac (*circles*) or skeletal (*squares*) SR was performed in the absence (defined as 100%) or in the presence of indicated concentrations of $IpTx_i$.

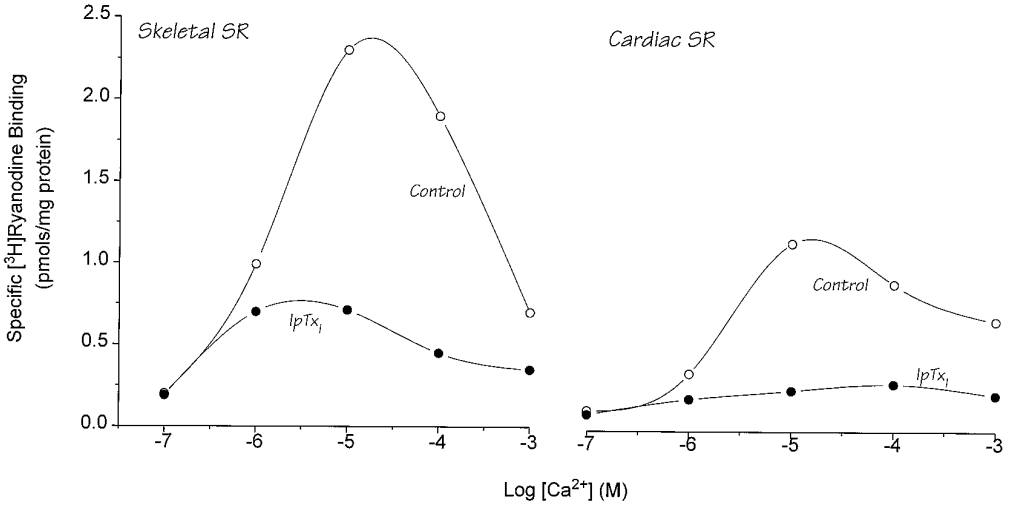

FIG. 6. **Ca^{2+} dependence of $IpTx_i$ inhibition of [^{3}H]ryanodine binding.** Rabbit skeletal (0.3 mg/ml) or pig cardiac (0.4 mg/ml) SR vesicles were incubated for 90 min at 36 °C with 7 nM [^{3}H]ryanodine in the absence (*open circles*) or the presence (*filled circles*) of 200 nM $IpTx_i$. The incubation medium consisted of 0.2 M KCl, 10 mM Na-Hepes (pH 7.2), 1 mM EGTA, and $CaCl_2$ necessary to bring free [Ca^{2+}] to the desired value. The Ca^{2+}:EGTA ratios were calculated by a computer program using affinity constants given in Fabiato (33).

cles—The membranes of SR vesicles contain different classes of phospholipids that may serve as substrates for the PLA_2 activity of $IpTx_i$. We reasoned that if the inhibitory properties of $IpTx_i$ were at least partly due to its enzymatic activity, then inhibition should be observed by incubating RyR with supernatant of $IpTx_i$-treated SR vesicles. Control SR vesicles were diluted in binding medium to 1 mg/ml and incubated at 36 °C with 100 nM $IpTx_i$. After 30 min, the $IpTx_i$-treated SR vesicles were pelleted at 32,000 × *g* for 15 min, and a clear supernatant was obtained. The supernatant was then tested for its capacity to inhibit the binding of [^{3}H]ryanodine to cardiac SR vesicles. A control tube that contained only $IpTx_i$ in binding medium without SR vesicles was run in parallel to determine the percentage of inhibition caused by the toxin alone. Fig. 7 shows that increasing concentrations of $IpTx_i$-treated SR supernatant inhibited the binding of [^{3}H]ryanodine dose-dependently (*filled circles*). This inhibition could not be attributed to $IpTx_i$ because the control supernatant reduced binding only marginally (*open circles*). For instance, in the most extreme case, 10 μl of $IpTx_i$-treated SR supernatant inhibited 100% of specific binding; the estimated concentration of $IpTx_i$ in this tube was 10 nM, which inhibited binding only by 22%. Hence, the lipolytic activity of $IpTx_i$ releases from SR vesicles a phospholipid product that is capable of inhibiting RyR activity.

Reversibility of $IpTx_i$ Effect—To rule out the possibility that inhibition of RyR by $IpTx_i$ was caused by an irreversible disruption of the channel protein, we incubated SR vesicles (1 mg/ml) with water (control) or with 1 μM $IpTx_i$ ($IpTx_i$-treated SR). After 30 min at 36 °C, an aliquot was taken from each sample. Then, both SR samples were washed twice, pelleted, and prepared for [^{3}H]ryanodine binding. Fig. 8 shows that binding of [^{3}H]ryanodine to $IpTx_i$-treated vesicles was decreased with respect to control after 30 min of incubation. However, these vesicles displayed essentially the same binding as control after washing off $IpTx_i$. Thus, RyRs recover their capacity to bind [^{3}H]ryanodine after treatment with $IpTx_i$. Assuming that the [^{3}H]ryanodine binding assay followed changes in channel gating (8–10, 17, 21), the changes described above suggest that $IpTx_i$ released a phospholipid product that bound to RyRs (or a closely associated regulatory protein) and decreased open probability (p_o); after removal of the phospholipid product, p_o could recover to control levels.

Single Channel Effects of $IpTx_i$—To test directly the above hypothesis and to gain insight on the manner in which $IpTx_i$ inhibits RyRs, we reconstituted swine cardiac RyR in planar lipid bilayers, as described (11, 17). Fig. 9 shows traces from continuous recording at −30 mV holding potential in the absence (control) and the presence of increasing concentrations of $IpTx_i$. Channel activity was monitored over 80 s in each condition, and the mean p_o was obtained from current histograms. The traces show that $IpTx_i$ blocks RyR dose-dependently: at low concentrations (≤200 nM) $IpTx_i$ decreases the lifetime of the open events, converting long openings into brief, frequently unresolved open events; at higher concentrations, both long and brief openings progressively decrease in frequency until they disappear. A quantitative description of this effect is presented in the open time histograms of Fig. 9*B*. In control, 2,034 events could be fitted with three exponentials with mean open time (τ) = 0.7 ms (66%), 1.9 ms (29%), and 9.8 ms (5%). In the presence of 500 nM $IpTx_i$, the histogram for 801 openings collected during the same period as control was monoexponential

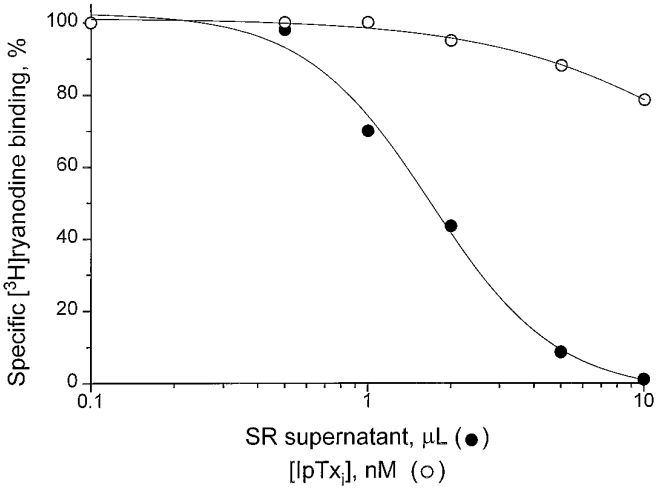

FIG. 7. **Inhibition of RyR by supernatant of $IpTx_i$-treated SR.** SR supernatant was prepared as described in the text. Binding of [^{3}H]ryanodine to cardiac SR vesicles was carried out in the absence (control, 100%) or presence of indicated volumes of SR supernatant (*filled symbols*). To keep the reaction volume constant, binding medium was added to SR supernatant to a final volume of 10 μl. To determine the inhibition caused by $IpTx_i$ alone, control vials containing $IpTx_i$ without SR vesicles were spun in parallel. The final concentration of $IpTx_i$ in the binding assays and the inhibition associated with it is shown by the *open symbols*.

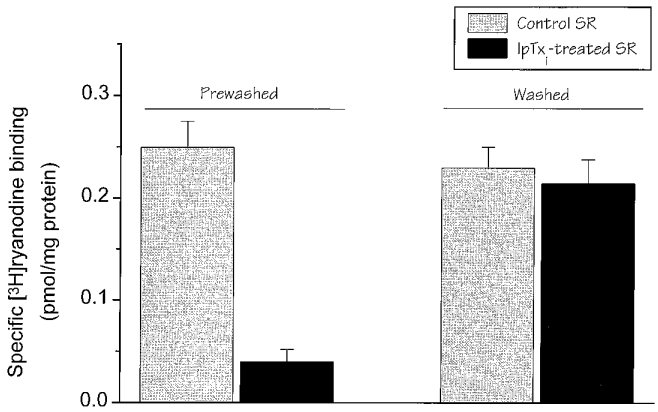

FIG. 8. **Reversibility of IpTx$_i$ effect.** Prewashed vesicles are cardiac SR vesicles incubated with water (control) or with 1 μM IpTx$_i$ (IpTx$_i$-treated SR) for 30 min at 36 °C. Washed vesicles are the same control and IpTx$_i$-treated vesicles after being pelleted and washed twice with binding medium. Binding of [^{3}H]ryanodine was conducted in all vesicles and is expressed in absolute values (pmol/mg).

with τ = 0.8 ms. Both the decrease in the frequency and duration of the open events contributed to the decrease in p_o. Fig. 9*C* shows the log dose-response relation of p_o as a function of IpTx$_i$ concentration. The IC_{50} for IpTx$_i$ inhibition was 140 nM. Since this value is in fairly good agreement with that determined in binding experiments (50–80 nM), this indicates that [^{3}H]ryanodine, at the concentrations used, does not interfere with the inhibitory capacity of IpTx$_i$. More importantly, the results show that the lipolytic product of IpTx$_i$ is capable of interacting directly with the RyR or with a closely associated protein that regulates channel gating.

Inhibition of RyR by Fatty Acids—PLA_2 catalyzes the hydrolysis of the S_n2 fatty acyl bond of phospholipids to release free fatty acids and lysophospholipids (22). We tested the effect of each of these lipids separately on RyR activity. Fig. 10*A* shows that lysophosphatidylcholine (lyso-PC), at concentrations up to 300 μM, does not modify substantially the open lifetime or the unitary conductance of cardiac RyR. On the other hand, linoleic acid, an 18-carbon unsaturated fatty acid, blocks RyR at lower concentrations and in a manner that is reminiscent of the blockade produced by IpTx$_i$. Addition of 30 μM linoleic acid to the cytosolic face of the channel decreases the lifetime and the frequency of open events; similar to IpTx$_i$, the fatty acid induces the appearance of openings that are too fast to be resolved by our recording bandwidth.

The potency of several fatty acids to inhibit RyRs was determined by their capacity to inhibit [^{3}H]ryanodine binding to cardiac SR vesicles (Fig. 10*B*). Palmitic acid, a 16-carbon saturated fatty acid, partially inhibited RyR but at concentrations $\geq$100 μM. Arachidonic acid, linoleic acid, and oleic acid, unsaturated fatty acids abundant in SR membranes of cardiac and skeletal muscle (23), totally inhibited RyR with an IC_{50} = 25, 55, and 70 μM, respectively. In agreement with single channel results, lyso-PC and lysophosphatidylethanolamine were unable to modify [^{3}H]ryanodine binding. Thus, not all PLA_2 products are capable of inhibiting RyRs. Among the fatty acids, the potency to inhibit RyRs increases with the carbon chain length and the number of unsaturations.

DISCUSSION

In the present study, we determined the complete amino acid and nucleotide sequence of IpTx$_i$, a heterodimeric protein from the venom of the scorpion *P. imperator*, and unraveled the molecular mechanism by which it inhibits RyRs of cardiac and skeletal muscle.

The deduced amino acid sequence from the cDNA encoding IpTx$_i$ and the amino acid sequence determined by Edman degradation strongly suggests that IpTx$_i$ is synthesized as a precursor of the pre-pro form. Putative signal and connector peptides must be removed to produce mature IpTx$_i$. The proposed signal peptide does have a large content of acidic residues (8 out of the last 18 residues are acidic), a property that is rarely seen in signal peptides. For this reason, we cannot exclude whether the proposed signal peptide codes for another peptide before coding for the large subunit of IpTx$_i$. Regarding the connecting pentapeptide Arg-Arg-Leu-Ala-Arg (positions 105 to 109 in the cDNA sequence of Fig. 3), we gather that several enzymes must be required to eliminate it from the mature protein. Initially, a cleavage must occur at Arg^{109} (monobasic site), as it occurs in the maturation of prosomatostatin and other prohormones (24). Two characteristics distinguish this cleavage site and both are present in the cDNA of IpTx$_i$: (i) Arg (or other basic residue) is immediately preceded by Ala or Leu (or both); (ii) Arg is located three or five residues upstream the basic amino acid involved in the cleavage. Next, another cleavage must occur at Arg^{106} (dibasic site) as is the case during the maturation of diverse peptidic hormones (25). A third enzyme with carboxypeptidase activity must intervene to remove Arg^{105} and Arg^{106}. Because of this complicated pattern of maturation, we assume that the linking of the small and large subunit of IpTx$_i$ must be important for the function of IpTx$_i$.

Mature IpTx$_i$ is composed of a ~3-kDa peptide covalently linked to a ~12-kDa peptide with PLA_2 activity. The large subunit of IpTx$_i$ conserves the most important substructures of secreted PLA_2 from group III (Fig. 4). The His/Asp pair, essential for Ca^{2+} binding, is in position 33/34 of IpTx$_i$ and is preceded in the three PLA_2s by a Cys-Cys-Arg motif; the amino terminus (roughly residues 4–12 of IpTx$_i$), presumably involved in substrate binding, is also highly conserved in the three PLA_2s; lastly, the Cys residues, essential for maintaining proper folding through disulfide bonds, may be used in this group as the elemental frame to align homologous sequences and to identify stretches of sequence that have been inserted or deleted through evolution. Only Cys^{101} of IpTx$_i$ (relative position 104 in Fig. 4) does not match with Cys residues present at the carboxyl terminus of the other PLA_2 shown. It is likely, therefore, that Cys^{101} is involved in the disulfide bridging with Cys^4 of the small subunit.

The presence in IpTx$_i$ of a PLA_2 subunit covalently linked to a small, structurally unrelated peptide is reminiscent of the bipartite arrangement of β-bungarotoxins (26), a group of snake neurotoxins that inhibit neurotransmitter release (27). Each β-bungarotoxin dimer has a PLA_2 subunit covalently bound to a smaller subunit related to Kunitz-type protease inhibitors. Members of the Kunitz superfamily bind to and block voltage-dependent K^+ and Ca^{2+} channels (28). Thus, although the small subunit of IpTx$_i$ showed no homology to members of the Kunitz superfamily, its distinctive arrangement within the toxin suggested that it might act as a blocker. For this reason, it was surprising at first to realize that the small subunit was not directly responsible for inhibiting RyRs. Direct addition of the small subunit to our [^{3}H]ryanodine binding assays failed to inhibit RyRs, as did a synthetic peptide with amino acid sequence similar to that of the small subunit and two additional derivatives designed to avoid interpeptide disulfide bridge formation (Table I). Hence, the target site for the small subunit, or its biological function, remains to be determined.

Given our failure to detect inhibition by the small subunit, we turned our attention to the large subunit of IpTx$_i$. In a reduced and carboxymethylated form, the large subunit of IpTx$_i$ failed to inhibit RyRs (not shown). However, this was not

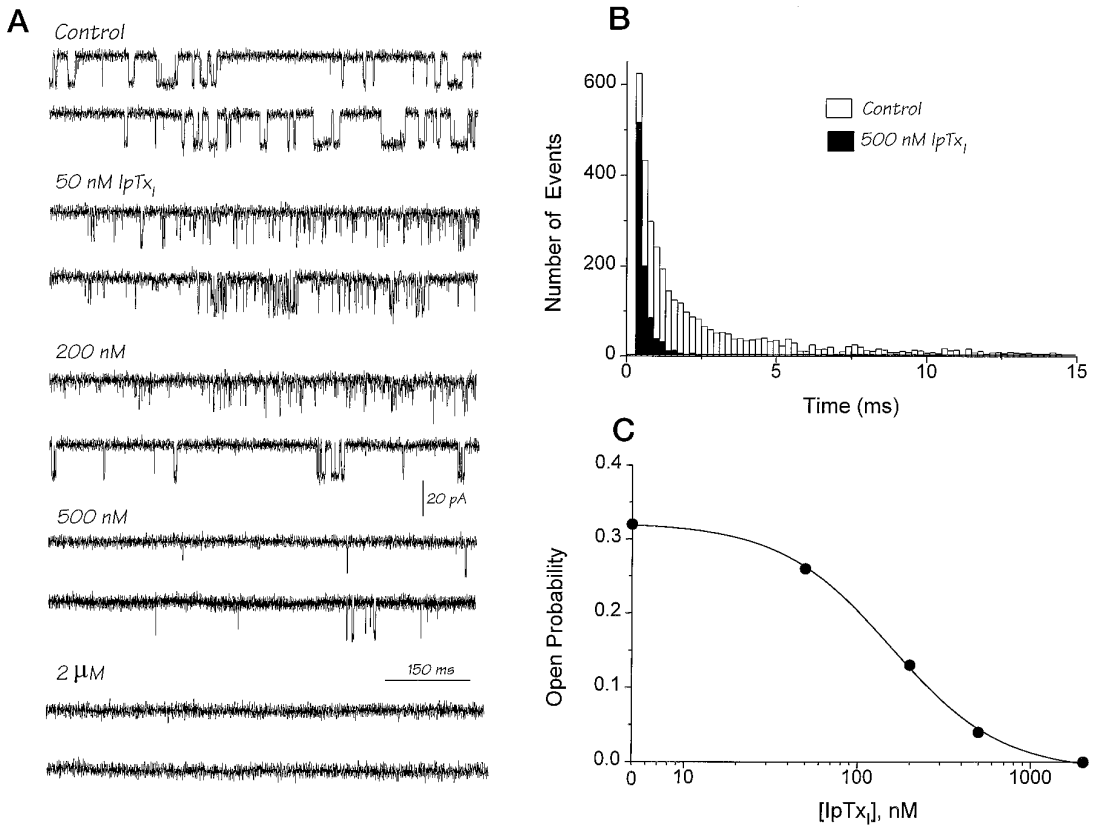

FIG. 9. **Inhibition of cardiac RyR activity by IpTx$_i$.** *A*, pig cardiac RyR were reconstituted in planar lipid bilayers and recorded in the absence (control) and the presence of indicated concentrations of IpTx$_i$. Holding potential: −30 mV. Recording solution: symmetrical 300 mM cesium methane sulfonate and 10 mM Na-Hepes (pH 7.2). Under these conditions, Cs^+ flows from the luminal (trans) to the cytosolic (cis) side of the channel, and openings are represented by downward deflections of the base-line current. *B*, open time histograms in the absence (*open bars*) and the presence (*filled bars*) of 500 nM IpTx$_i$. *C*, plot of open probability (P_o) as a function of [IpTx$_i$]. *Smooth line* is a fit to data points using the expression $p_o = (p_o \text{ control})/1 + ((\text{IpTx}_i)/\text{IC}_{50})^{n_H}$. Where p_o control is the p_o in the absence of IpTx$_i$ (0.32); IC_{50} is the concentration of IpTx$_i$ that produces half-maximal inhibition (140 nM); and n_H is the Hill coefficient (1.22).

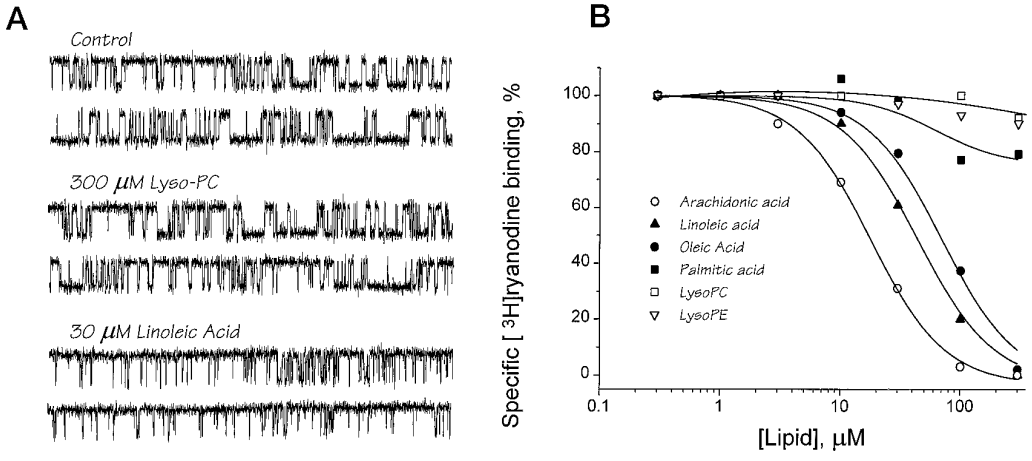

FIG. 10. **Selective inhibition of RyR by fatty acids.** *A*, representative traces of cardiac RyR activity in control (*top trace*) and in the presence of 300 μM lyso-PC (*middle trace*) or 30 μM linoleic acid (*bottom trace*). Openings are represented by downward deflections of the base-line current. For the experiment shown plus two additional experiments, mean p_o was 0.43 ± 0.09 (control), 0.46 ± 0.11 (lyso-PC), and 0.12 ± 0.06 (linoleic acid). *B*, the binding of 7 nM [^{3}H]ryanodine to cardiac SR vesicles was determined in the presence of methanol (maximal concentration = 1%) and this value defined as 100%, or in the presence of indicated concentrations of lipids dissolved in methanol.

surprising given that disulfide bridges are essential to maintain the toxin's three-dimensional structure (20). It was the treatment of IpTx$_i$ with *p*BPB, a covalent modifier of His residues that inhibits PLA_2 activity without affecting disulfide bridges (15), that decreased substantially the capacity of IpTx$_i$ to inhibit RyRs (Fig. 5). This suggested that a lipid product of PLA_2 activity was involved in the inhibition of RyRs. Three separate lines of evidence supported this notion. 1) Inhibition of RyR by IpTx$_i$ was favored by the presence of Ca^{2+} (≥1 μM; Fig. 6), as expected from the Ca^{2+} dependence of enzymatic activity of PLA_2 from group III and several other groups (20). 2) The supernatant of IpTx$_i$-treated SR vesicles could inhibit RyRs even before IpTx$_i$ was present in a concentration large enough to do so (Fig. 7); this indicated that RyR inhibition was brought

about by a lipid product of $IpTx_i$ released from SR vesicles during the incubation period. 3) The kinetics of RyR inhibition by $IpTx_i$ were mimicked by direct addition of linoleic acid (but not of lyso-PC) to the cytoplasmic side of the channel (Figs. 9 and 10); other long chain, unsaturated fatty acids could also inhibit RyRs (Fig. 10). Thus, a potential scenario for inhibition of RyR by $IpTx_i$ is as follows: in the presence of micromolar [Ca^{2+}], the large subunit of $IpTx_i$ catalyzes the hydrolysis of the S_n2 fatty acyl bond of the phospholipids of the SR membrane. The reaction yields free fatty acids and lysophospholipids; the former are released into the incubation medium, and the latter may be liberated or remain embedded into the SR membrane. Free fatty acids bind to RyR or to a closely associated protein that controls gating. At low concentrations, they produce an incomplete block of RyR; higher concentrations gradually block the RyR completely, giving rise to the dose-response relationship of channel activity and [^{3}H]ryanodine binding *versus* $IpTx_i$ concentration of Figs. 1, 5, and 9.

PLA_2s are abundant components of snakes, scorpions, and bee venoms and often constitute the main toxic component to mammals. Although all PLA_2s induce a variety of pathological symptoms including neurotoxicity and myotoxicity (29), they differ in their mechanism of action and in their molecular targets. For example, the snake neurotoxin crotoxin is composed of a PLA_2 subunit and an inhibitory subunit, which keeps the phospholipase inactive until binding to a presynaptic receptor triggers the dissociation of the inhibitory subunit (30). In contrast, notexin from *Netechis scutatus scutatus* is a PLA_2 without an associated subunit that produces muscle paralysis by binding to a specific receptor in the neuromuscular junction (31). $IpTx_i$ is the first example of a scorpion toxin in which a PLA_2 is found chaperoned by a smaller, structurally unrelated subunit. In a previous study (11), we found that several transporters and ion channels of striated muscle including the inositol trisphosphate receptor, the muscarinic receptor, voltage-sensitive Na^+ channels, and the Ca^{2+}-ATPase of SR were insensitive to micromolar concentrations of $IpTx_i$. These results argue against an indiscriminate effect on key molecules of excitation-contraction coupling by the lipolytic action of $IpTx_i$. However, an important question that still remains to be answered concerns the presence of the small subunit of $IpTx_i$. What is its molecular target? What function does it perform to enhance the PLA_2 activity or to suppress it while in transit to the specific receptor? Regardless of the primary site affected, a major contribution of this study was to establish the fact that the molecular mechanism involved in the toxicity of $IpTx_i$ will most likely involve abnormalities in intracellular Ca^{2+} mobilization due to blockade of RyRs.

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Phospholipin, a novel heterodimeric phospholipase A2 from *Pandinus imperator* scorpion venom

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Abstract The primary structure of a phospholipase A2, with unique structural and functional characteristics, was determined. The large subunit has 108 amino acid residues, linked by a disulfide bridge to the small subunit, which contains 17 residues. Its gene was cloned from a cDNA library. The nucleotide sequence showed that the same RNA messenger encodes both subunits, separated only by a pentapeptide, that is processed during maturation.

Key words: Phospholipase A2; Amino acid sequence; Nucleotide sequence; Scorpion venom; *Pandinus imperator*

1. Introduction

Phospholipase A2 (PLA2) cleaves the ester bond at position 2 of the glycerol moiety of phospholipids, and is widely distributed in mammals, reptiles and arthropods [1]. Recently, the old classification of PLA2 into three groups [2] was expanded according to some structural and functional features, such as the number of disulfide bridges and the kind of amino acids responsible for the catalytic activity, found in the newly discovered PLA2 [3]. Dennis [3] reports seven well-defined PLA2 groups (I–VII) and adds three additional ones (VIII–IX), not fully characterized. Apart from the general hydrolytic function of these enzymes, several specific biological functions have been associated with members of the PLA2 superfamily. Among these functions are anti-inflammatory action [4], myonecrotic and muscle damaging effects [5] and ion channel blocking activity [6]. β-Bungarotoxin and crotoxin are two other well known toxic venom components with PLA2 activity [7,8]. In the venom of the scorpion *Pandinus imperator* an interesting heterodimeric phospholipase (IpTxi) was recently described. This protein causes inhibition of ryanodine binding to the Ca^{2+} channels present in skeletal muscle, very likely due to an indirect effect caused by the fatty acid liberated by the PLA2 activity of IpTxi [6].

In this communication we report the isolation and characterization of a novel heterodimeric protein from the venom of the same African scorpion *P. imperator*. The large subunit shares sequence similarities with the phospholipase moiety of IpTxi [6] and with those of phospholipases from honeybee and *Heloderma* lizard, but has very little or no effect on the inhibition of ryanodine binding to Ca^{2+} channels. The small subunit is unique to this heterodimeric phospholipase. We propose to call it phospholipin, and assume that together with IpTxi it will constitute a new group of PLA2, number X, following the classification proposed by Dennis [2].

2. Materials and methods

2.1. Chemicals and purification procedure

All chemicals were analytical grade reagents, obtained from sources already described [6]. The venom of the scorpion was obtained in the laboratory by electrical stimulation, dissolved in double-distilled water, centrifuged at $15\,000\times g$ for 15 min and the supernatant lyophilized. The soluble venom applied to a Sephadex G-50 column (0.9×190 cm) in 20 mM ammonium acetate buffer, pH 4.7, resolved at least five fractions, of which number II contains phospholipase activity; this fraction was further separated into a carboxymethyl-cellulose (CM-cellulose) column, as described earlier for IpTxi [6]. One of the sub-fractions from CM-cellulose (number 3) contains phospholipin, which was finally obtained in homogeneous form by high performance liquid chromatography (HPLC), using a C18 reverse-phase column (Vydac, Hisperia, CA).

2.2. Phospholipase assay and determination of specificity

The egg yolk-agarose system of Habermann and Hardt [9] was used to follow the presence of phospholipase activity during the purification procedure. The enzyme specificity was determined using radioactively labeled substrates, and thin layer chromatography, as described previously [10].

2.3. Amino acid analysis and microsequencing

Amino acid analysis was performed in samples hydrolyzed in 6 N HCl with 0.5% phenol at 110°C in evacuated, sealed tubes as described [6]. Reduced and alkylated phospholipin, in amounts of 100 μg each time, was cleaved independently by three different enzymes, and the corresponding peptides were separated by HPLC, using the conditions described in the legend for Fig. 1. Digestion with *Staphylococcus aureus* protease V8 was performed in 100 mM ammonium bicarbonate buffer, pH 7.8, for 4 h, at 40°C, whereas hydrolysis with endopeptidases AspN and ArgC was performed in the conditions described elsewhere [11]. Microsequence determination was performed on a 6400/6600 Milligen/Biosearch Prosequencer, using the peptide adsorbed protocol on CD Immobilon membranes [11].

2.4. Mass spectrometry determination

The molecular weight of pure phospholipin was determined by mass spectrometry, using a Kratos Kompact MALDI 3 v. 3.0.2 apparatus.

2.5. Cloning and sequencing

Two degenerated oligonucleotides encoding two different regions of phospholipin were synthesized, as previously described [6].

Oligo 1 (ATG TGG GAR TGY ACN AAR TGG TG-, where N is any nucleotide, R is A or G and Y is C or T-) corresponds to the DNA sequence of amino acids 2–10 of the large subunit, whereas oligo 2 (TGY GAR AAY GGN GTN GCN AC-) corresponds to the DNA sequence of amino acids 4–11 of the small subunit.

Total RNA was purified as reported [6]. Messenger RNA was purified following the instructions of the Hybond mAP protocol (messenger affinity paper; Amersham, RPN.1511). Synthesis of cDNA and the cloning of the cDNA library was performed as described [6,11]. The screening of the library was performed separately with oligonucleotides 1 and 2. The clone detected with oligonucleotide 1 was analyzed first. Inserts of cDNA from positive clones were amplified by polymerase chain reaction (PCR) using λ gt11 forward and reverse

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primers. PCR products were subcloned into pBluescript (pKS) phagemid. Clones of interest were sequenced using the Sequenase kit v. 2.0. (U.S. Biochemical Corp.). Oligos M13-20 and M13 reverse were used for sequencing [11].

3. Results and discussion

Fig. 1 shows the separation of soluble venom from *P. imperator*, by Sephadex G-50, CM-cellulose and HPLC. Fraction II (Fig. 1A) contains phospholipase activity. Sub-fraction 1 in Fig. 1B corresponds to IpTxi, capable of inhibiting ryanodine binding to skeletal muscle Ca^{2+} channels, as described by Zamudio et al. [6], whereas sub-fraction 3 (Fig. 1B) also contains a protein with phospholipase activity, which can be further purified by HPLC (inset Fig. 1B). The major component from the HPLC chromatogram (shown by the asterisk in the inset of Fig. 1) was assumed to be homogeneous, based on results obtained from SDS-gel electrophoresis (data not shown). It contains phospholipase activity but very little, if any, action on the inhibition of ryanodine binding to its receptor, contrary to IpTxi (data not shown). For this reason it was called phospholipin, without any additional reference to the ryanodine-sensitive Ca^{2+} channels. Mass spectrometry analysis showed that phospholipin was homogeneous, with a molecular mass of 14 841.2. Based on this analysis, the molecule was expected to contain in the order of 125 amino acid residues. When this protein was loaded into the microsequencer, two amino acid residues of about the same amount showed in each cycle, suggesting that it consisted of a heterodimeric protein. Reduction and alkylation of phospholipin permitted separation of the two subunits. This was obtained by gel filtration on a Bio-gel P-30 column, using the same system described for IpTxi [6]. Each one of the subunits was separately sequenced. Fig. 2A shows the results obtained. The short subunit was directly sequenced to the end, and contains only 17 amino acid residues, in which position 4 is occupied by a cysteine, responsible for the covalent attachment of the short peptide to the long one, through a disulfide bond. The larger subunit is composed of 108 amino acid residues and its complete sequence was determined after enzymatic cleavage. The sub-peptides were separated by HPLC (data not shown) and the overlapping sequences obtained are shown in Fig. 2A, underlining the overall amino acid sequence. Direct sequencing permitted the identification of the first 22 amino acid residues, whereas four sub-peptides obtained by cleaving with *S. aureus* protease V8 gave segments from amino acid residues in positions 20–37, 65–71, 73–88 and 101–108. The protease V8 cleavage was partial, since some of these fragments still contained internally situated glutamic acid residues, supposed to be cleaved by this enzyme, when the hydrolysis goes to completion. Additional positioning of residues was obtained by the use of two other proteinases. They correspond to the segment at positions 38–59 obtained by means of AspN protease hydrolysis, and sub-peptides 47–64 and 89–101, obtained by ArgC protease cleavage. It is worth mentioning that the specificity of ArgC was not 100% either; the batch of enzyme we used was able to cleave a lysine residue in position 46. The overall sequence determined by direct Edman degradation of the sub-peptides of phospholipin was additionally confirmed by the nucleotide sequence obtained from the cloned gene. The obtained cDNA nucleotide sequence of phospholipin is shown in Fig. 2B. The first gene cloned was performed using oligo 1 (see Section 2), designed to recognize the large subunit sequence, and the second was oligo 2. When the first cloned gene, based on the amino acid sequence of the large subunit (cloned with the help of oligo 1), was hybridized with oligo 2, specific for the short peptide, it was shown that both oligos were capable of recognizing the same clone. Under DNA sequencing analysis this initially misleading result was immediately understood. The gene that encodes both peptides is transcribed into the same mRNA, which encodes the large and the small subunit as well, separated by a pentapeptide Lys-Arg-Ser-Gly-Arg, which is processed during maturation, in quite the same way as initially demonstrated for the case of IpTxi, by our group [6]. The analysis and interpretation of Fig. 2B is still not complete, because there is a long 5′ non-translated segment of the cloned gene, whose function is still not certain. How-

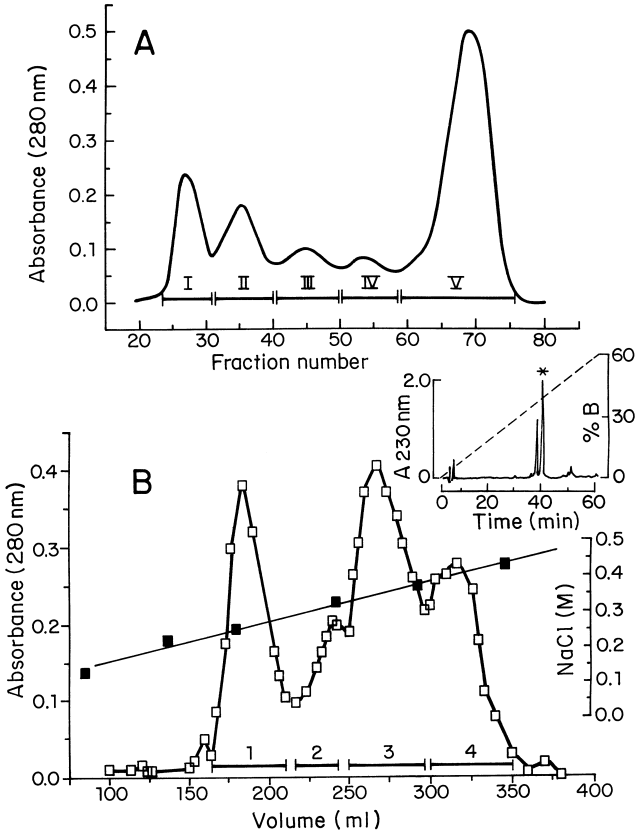

Fig. 1. Purification of phospholipin. A: Soluble venom from *P. imperator* (120 mg of protein) was applied to a Sephadex G-50 column (0.9×190 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7. Fractions of 5 ml each were collected and tested for their phospholipase activity. B: Tubes corresponding to fraction II were enzymatically active and were further applied to a CM-cellulose chromatographic column (0.9×30 cm) dissolved in the same buffer. The column was eluted with a linear gradient of sodium chloride, as indicated. Fractions 1 and 3 displayed phospholipase activity; the first contained IpTxi [6], whereas number 3 was finally separated by HPLC in a C18 reverse-phase column, as shown in the inset. Phospholipin corresponds to the major, last sub-fraction, labeled with an asterisk. A linear gradient was used from solvent A (0.12% trifluoroacetic acid in water) to 60% B (0.10% trifluoroacetic acid in acetonitrile). Phospholipin corresponds to approximately 2.4% of the soluble venom. The experiments aimed at determining its specificity, using radiolabeled phospholipids, clearly showed that phospholipin has phospholipase A2 type activity (results not shown).

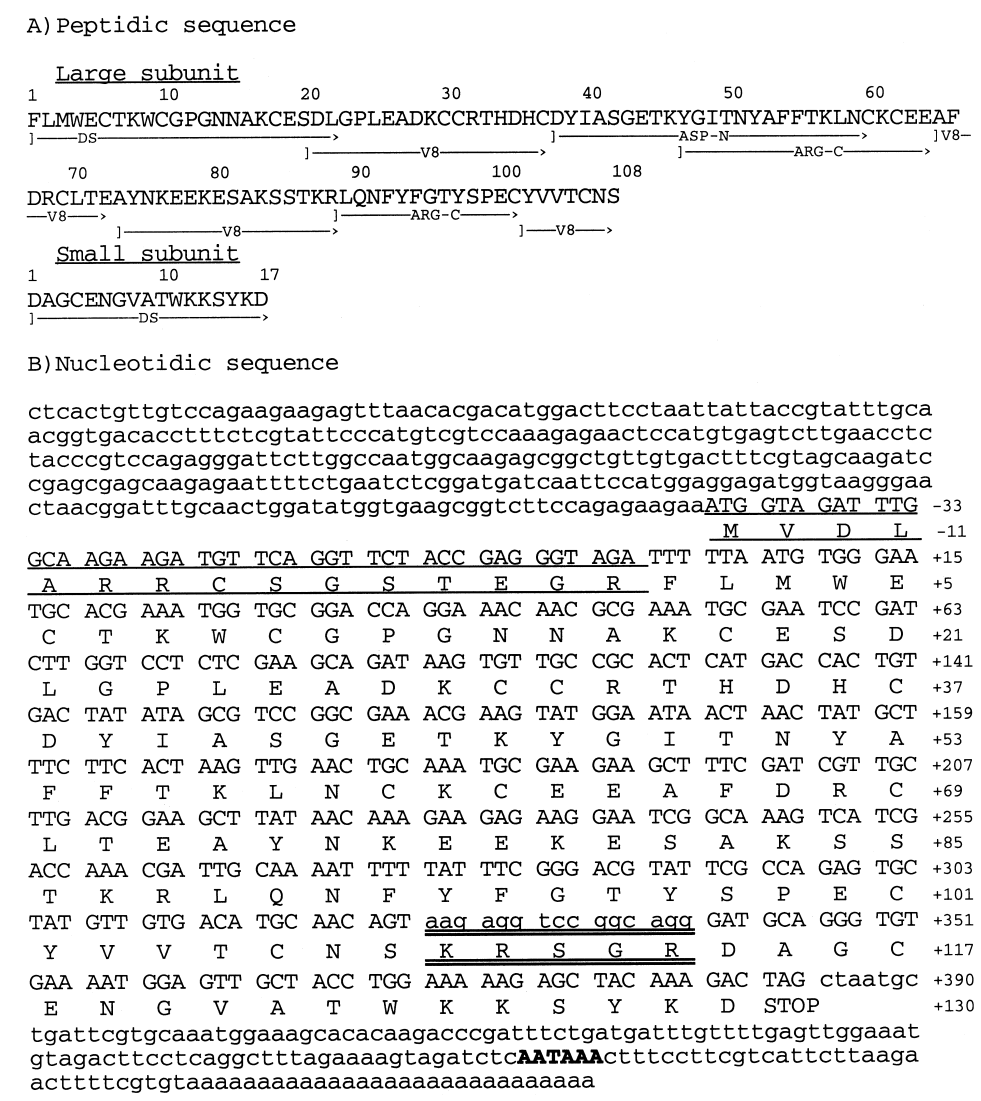

Fig. 2. Amino acid and nucleotide sequence of phospholipin. A: Primary structure of phospholipin as determined by direct sequencing of the native peptides (underlined DS) and fragments produced by enzymatic hydrolysis with protease V8 (labeled V8), endopeptidase AspN (labeled ASP-N) and endopeptidase ArgC (labeled ARG-C). B: Nucleotide sequence from the 5′ to the 3′ end of the clone that encodes phospholipin. The sequence corresponding to the putative signal is underlined, the pentapeptide eliminated during processing is double underlined, bold letters indicate the possible polyadenylation site, whereas lower case letters indicae the 5′ and 3′ non-coding regions of the gene. Numbers on the right hand side correspond to the positions of nucleotides from the signal peptide to the stop codon (upper lines) and to their corresponding amino acids (lower lines).

ever, the sequence that encodes the heterodimer phospholipin molecule is very clear. It is composed of 393 nucleotides (including the stop codon), starting at residue 1 with Phe (see line 8 in Fig. 2B) labeled on the right hand side with number +5 (which corresponds to glutamic acid), and ends at residue 130, just before the stop codon. In this sequence there is a pentapeptide (double underlined) that is processed during maturation, because it was not seen when we sequenced the heterodimeric peptides. There is a putative signal peptide (single underlined in positions −15 to 0), but there are still 304 nucleotides to the left side, with unknown function. That is, the long 5′ non-translated sequence (lower case letters) could contain information for a longer signal peptide, that might start at the ATG codon situated 9 residues to the left of the first Met residue (our amino acid −15 in Fig. 2B) implying that the signal peptide could start at amino acid −24. It is also not clear if the putative signal peptide we have labeled is, in fact, the signal peptide. We are concerned about the presence of two Arg residues within this segment. Thus, the possibility that this segment corresponds to the message for translating an unknown third peptide or long propeptide needs further analysis and work. Also, the long 3′ non-translated region (104 nucleotides), before the putative polyadenylation site (bold capital letters at the end of the figure) needs some additional work.

Although the primary structure of the large subunit of phospholipin presents a close similarity with PLA2 from honey bee and *Heloderma* lizard, which would place it in the group III phospholipases, it is not certain that it fits there. One of the main reasons is because it is a heterodimer in which the small subunit (17 amino acid residues) has no resemblance to any other protein known. It seems clear that the large subunit moiety is the one bearing the phospholipase activity, in all three examples mentioned. Also β-bungarotox-

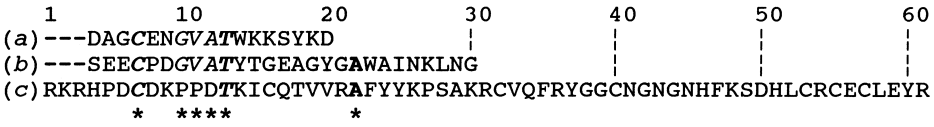

Fig. 3. Comparative analysis of the small phospholipase subunits. The amino acid sequence of the small subunits of phospholipin (this work), and those of IpTxi [6] and β2-bungarotoxin [13] were compared, after alignment of the first cysteinyl residues by adding gaps (-) to the sequences of phospholipin and IpTxi. Very little identity exists. Phospholipin (a) and IpTxi (b) share five identical amino acid residues at the same position (18% identity), whereas with the small β1 subunit of bungarotoxin (c) only two positions were occupied by identical residues (less than 4% identity). Bold italics indicate identical residues in all three sequences, italics indicate identity for only two of the sequences on pairwise comparison. Identical residues are highlighted by asterisks.

in, a presynaptically active phospholipase isolated from the snake *Bungarus multicinctus* [7], and IpTxi, isolated from the scorpion *P. imperator* [6] are heterodimers, but again there is no resemblance in structure or function, when we compare the small subunits of these three venom components, as shown in Fig. 3. Also functionally, the short peptide of β-bungarotoxin with 61 amino acid residues was suggested to be a K^+ channel blocker [12], whereas that of IpTxi (27 amino acid residues) apparently has no measurable function towards Ca*2*+ channels or towards any other of several ion channels assayed (see [6]). Phospholipin has the shortest of all the small subunit peptides of the three heterodimeric phospholipase known, and we do not know either what its function is. The three-dimensional structure of phospholipin was modelled using the Swissmod program and facilities (data not shown), and the results of the model suggests that cysteine at position 4 of this 17 amino acid peptide is bound to cysteine in position 101 of the phospholipase moiety. Structurally, phospholipin shows two additional cysteines (one more disulfide bridge), not present in IpTxi, which might be responsible for folding phospholipin in a different manner than that of IpTxi. It can be speculated that this structural difference is responsible for a distinct substrate specificity, hence a different biological action, such as the indirect effect on the binding of ryanodine to the Ca^{2+} channels, described for IpTxi. Due to these characteristics, phospholipin described here, although similar to group III phospholipases A2, seems to constitute the prototype of a novel group of PLA2, which would be number X, according to the classification recently proposed by Dennis [3].

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Scorpine, an anti-malaria and anti-bacterial agent purified from scorpion venom

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Abstract A novel peptide, scorpine, was isolated from the venom of the scorpion *Pandinus imperator*, with anti-bacterial activity and a potent inhibitory effect on the ookinete (ED_{50} 0.7 μM) and gamete (ED_{50} 10 μM) stages of *Plasmodium berghei* development. It has 75 amino acids, three disulfide bridges with a molecular mass of 8350 Da. Scorpine has a unique amino acid sequence, similar only to some cecropins in its N-terminal segment and to some defensins in its C-terminal region. Its gene was cloned from a cDNA library.

Key words: Antibiotic; Malaria; Scorpine; *Pandinus imperator*; *Plasmodium*

1. Introduction

Scorpion venoms are rich sources of peptides with a variety of pharmacological functions, specially those that affect membrane permeability for Na^+, K^+, Ca^{2+} and Cl^- of excitable and non-excitable cells (review [1]). They have been excellent tools to study ion-channel structure and function [2]. However, these venoms contain other interesting peptides, such as phospholipin [3] and imperatoxin-I [4], both with phospholipase activity, and the anti-microbial and anti-malaria peptide described here. Other peptides that could be effective in the control of malaria, a parasitic disease caused by *Plasmodium spp* and transmitted to vertebrates by mosquitoes, were also investigated lately [5,6]. The long range aim of the work is to incorporate into mosquitoes the genes encoding the bioactive peptides to produce transgenic vectors resistant to malaria [7]. Among the peptides isolated from the venom of the African scorpion *Pandinus imperator*, we identified a molecule, named scorpine. Due to the hybrid similarity of this peptide with the amino acid sequence of known cecropins and defensins [8], the idea came of assaying its effect on microbial growth and *Plasmodium berghei* stages that develop in mosquitoes. The description of the structure and biological function of scorpine is the object of this communication.

2. Materials and methods

2.1. Chemicals and purification procedure

All chemicals were analytical grade reagents, obtained from sources already described [3,4]. The venom of the scorpion was obtained in the laboratory by electrical stimulation, and prepared for chromatographic separations as previously reported [4]. The soluble venom applied to a Sephadex G-50 column (0.9×190 cm) in 20 mM ammonium acetate buffer, pH 4.7, resolved five well-defined fractions, from which fraction number II was subsequently applied to a carboxymethyl-cellulose (CM-cellulose) column, as described earlier for the isolation of imperatoxin-I [3] and phospholipin [4]. Scorpine was finally obtained in homogeneous form by high performance liquid chromatography (HPLC) separation, using a C18 reverse-phase column (Vydac, Hisperia, CA, USA).

2.2. Amino acid sequence

Scorpine was sequenced either as a native peptide or after reduction and alkylation, using the method described [3,4]. An aliquot (100 μg) of alkylated peptide was cleaved with endopeptidase AspN and separated by HPLC (data not shown), following the conditions described elsewhere [4]. Each independent sub-peptide was placed into the sequencer for obtaining overlapping amino acid sequences. Microsequence determination was performed on a 6400/6600 Milligen/Biosearch prosequencer, using the peptide adsorbed protocol on CD Immobilon membranes [3,4].

2.3. Mass spectrometry determination

The molecular weight of pure scorpine was determined by mass spectrometry, using a Kratos Kompact MALDI 3 V 3.0.2, apparatus.

2.4. Cloning and sequencing

Isolation of RNA and preparation of a cDNA library was performed as described [9]. The screening of the library was performed using a degenerated oligonucleotide (ATG GCN AAY ATG GAY ATG CT, where N means any nucleotide and Y means T or C), which encodes for the amino acid sequence of residues 40 to 46 (Met-Ala-Asn-Met-Asp-Met-Leu) of scorpine, synthesized as previously described [6]. The clone detected with this oligonucleotide was amplified by polymerase chain reaction (PCR) using (lambda) gt11 forward and reverse primers. PCR products were subcloned into pBluescript (pKS) phagemid and sequenced using the Sequenase® kit V. 2.0. (US Biochemical corp.). Oligo M13-20 and M13 reverse were used for sequencing [9].

2.5. Anti-bacterial assays

The classical method of Fleming was used for testing the anti-bacterial activity of scorpine. *Escherichia coli* ATCC 25922, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Klebsiella pneumoniae*, were initially assayed. Inhibition zones were recorded around wells in thin agar plates with bacteria, as described by Hultmark et al. [15].

2.6. Parasites

The gametocyte-producing clone of *P. berghei* Anka strain 2.34 (kindly provided by R.S. Sinden, Imperial College of Science Technology and Medicine, UK) was used. Parasites were maintained in BALB/c mice by mechanical passage. Mice, with parasitemias ranging between 50 and 60% and gametocytemia around 10% were bled by heart puncture using heparin (100 IU/ml blood). This usually occurred after 8–10 days post-infection.

2.7. Susceptibility of P. berghei sexual stages to scorpine

Ookinete cultures were carried out as described [5]. Leucocyte-de-

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pleted infected-mouse blood was suspended 1:5 in culture medium and tested in 100 μl aliquots in flat-bottom 96-well plates. Scorpine at concentrations of 0.049 to 100 μM was added to triplicate wells and the number of rosettes, indicative of active fecundation, was assessed by bright field microscopy between 10 and 30 min. later. The number of ookinetes were assessed 24 h later in Giemsa-stained blood smears.

3. Results and discussion

Fig. 1 shows the profile of CM-Cellulose separation of fraction 5, from the soluble venom of *P. imperator* after gel filtration on Sephadex G-50 [4]. This chromatogram shows a better resolution of that earlier reported [4], because it was applied in a much greater amount of protein and separated with a slightly different gradient. The component eluting at about 0.35 M NaCl (labeled with an asterisk) was further applied to a C18 reverse phase column providing homogeneous scorpine (marked with an asterisk in the inset-figure). Small contaminants with weak phospholipase activity were eliminated by this HPLC step. Scorpine purified by this procedure corresponds to approximately 1.4% of the total venom.

Direct automatic Edman degradation of native scorpine permitted the identification of the first 38 amino acid residues of the N-terminal segment, whereas a sample of scorpine reduced and carboxymethylated, permitted the unequivocal assignment of the first 47 amino acid residues of the primary structure, as shown in Fig. 2A. Two additional sub-peptides obtained by HPLC, after digestion with AspN endopeptidase (data not shown), allowed the identification of the C-terminal part of the molecule (residues in position 44–75), as well as an extended overlapping region of scorpine (residues from positions 14 to 43). Additional segments with excellent overlapping positions were also obtained, but not included in Fig. 2A, for simplification of the picture. The completion of the sequence was confirmed by the results of molecular weight determination using mass spectrometry analysis. The molecular mass of scorpine, 8350 is compatible both with the direct amino acid sequence obtained from the sequencer, and that deduced from the cloned gene. Fig. 2B shows the nucleotide sequence of a gene cloned using the internal oligonucleotide for hybridization and the (lambda) gt11 forward and reverse primers for amplification. A clone containing 487 nucleotides was sequenced. The internal segment contains the information (indicated by capital letters) that encodes the amino acids in position 1–75 of mature scorpine, ending in the stop codon at position 76. Two additional stretches of nucleotides were also found, one situated at the 5′ region, where a putative signal peptide containing the information for 19 amino acids is shown, and another at the 3′ untranslated region, where a putative adenylation site was identified (see lower case letters in bold, in Fig. 2B).

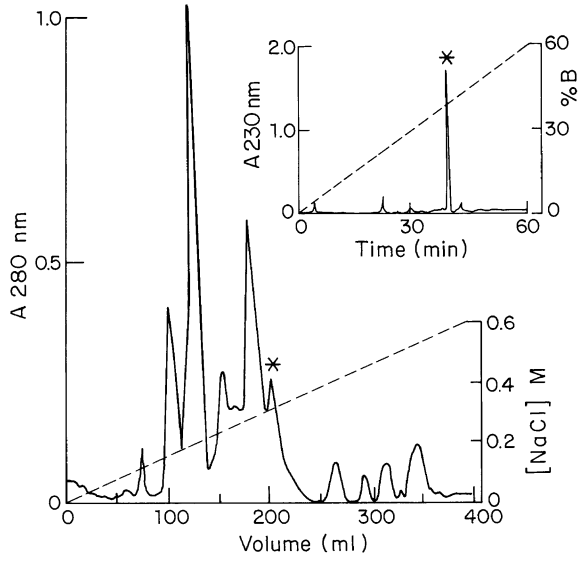

Fig. 1. Purification of scorpine. Fraction II (63 mg) of the venom from *P. imperator* separated by Sephadex G-50 gel filtration (see figure 1 in [4]) was loaded into a CM-cellulose chromatographic column (0.9×30 cm) dissolved in 20 mM ammonium acetate buffer, pH 4.7. The column was eluted with a linear gradient of sodium chloride, from 0.0 to 0.6 M salt. The fraction labeled with an asterisk contained anti-bacterial activity, and some residual phospholipase activity and was further purified by HPLC in a C18 reverse-phase column, as shown in the inset. Scorpine corresponds to the major component, labeled with an asterisk. A linear gradient was used from solvent A (0.12% trifluoroacetic acid in water) to 60% B (0.10% trifluoroacetic acid in acetonitrile).

When the amino acid sequence of scorpine was compared with other known peptides, using data banks (SwissProt and FASTA program), none of them showed substantial similarities to that of scorpine, except for a small stretch of the C-terminal segment, that contains three-disulfide bridges like those of defensins [10,11]. Also, the N-terminal region was similar to cecropins [12–14]. Fig. 3C shows the comparison of the primary structure of scorpine with these peptides (only representative examples were chosen for this figure). It is clear that scorpine is a unique peptide, much longer than other anti-bacterial peptides (practically double size). It seems to be a hybrid of cecropin and defensin, showing some identical amino acids (labeled with an asterisk in Fig. 2C) in certain stretches of the sequence, when appropriate gaps are manually introduced in order to increase similarities.

Due to the fact that the N-terminal segment of scorpine showed similarity to cecropins, the first idea was to test its effect against bacteria.

The method described by Hultmark et al. [15] was used to access the effect of scorpine on the inhibition of bacterial growth. Clear inhibition was obtained for *B. subtilis* (Minimum inhibitory concentration (MIC) around 1.0 μM) and for *K. pneumoniae* (MIC of approximately 10 μM), respectively.

The biological function of scorpine in *P. imperatur* awaits investigation. It is possible that it may function as microbicide within the venom gland which is open to the exterior and in contact with the hemocele of the scorpion prey during the sting.

Transmission of malaria parasites by mosquito vectors is dependent on the successful development of *Plasmodium* infective forms following ingestion of a blood-meal infected with gametocytes. This process is complex and includes a series of morphological and physiological transformations within the mosquito midgut (gametogenesis, fecundation, and ookinete formation) [16]. We previously observed in in vitro and in vivo tests that 100 μM Shiva-3, a synthetic cecropin, inhibited *P. berghei* ookinetes development [5–7]. The similarity of scorpine to cecropin prompted us to investigate its effect on the same *P. berghei* sexual stages. Our results showed that scorpine completely inhibited both fecundation and ookinete formation at 50 and 3 μM, respectively (Fig. 3), and that it had a toxic effect on gametes and on ookinetes at

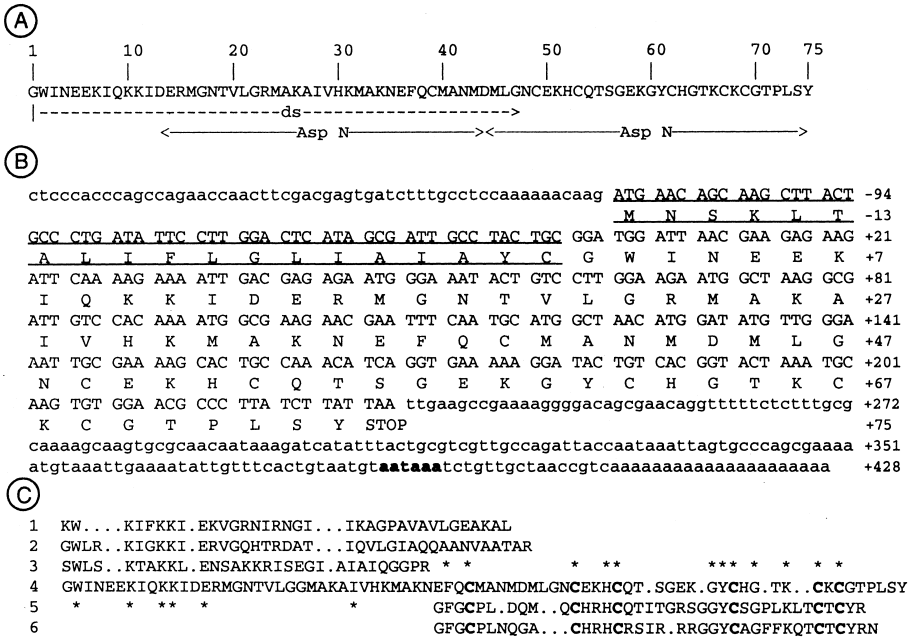

Fig. 2. Amino acid and nucleotide sequence of scorpine. A: Primary structure of scorpine as determined by direct sequencing the native peptides (underlined DS) and fragments produced by enzymatic hydrolysis with endopeptidase AspN (labeled ASP-N). B: Nucleotide sequence written from the 5′ to the 3′ end of the clone that encodes scorpine. The sequence corresponding to the putative signal is underlined. The mature peptide is indicated from residues 1–75, ending with the stop codon. The lower case letter in bold at the 3′ region indicates the possible poly-adenylation site, whereas lower case letters at the extreme 5′ and 3′ ends the non-coding region of the gene. Numbers on the right side of the page correspond to the positions of nucleotides from the signal peptide to the stop codon (upper lines) and to their corresponding amino acids (lower lines). C: Amino acid sequence comparison of scorpine with that of cecropin and defensin antibiotic peptides. The sequences were aligned with extended blank spaces, because of the difference in size of the peptides and lack of extended similarities. The segments with some consensus sequences are shown pairwise with artificial blank spaces (small dots, to enhance similarity) and whenever an identical residue is situated in equivalent position, an asterisk is placed either down or up to the sequence of scorpine. The sequences compared are: cecropin B from *Antheraea pernyi* [12]; sarcotoxin Ic from *Sarcophaga peregrina* [13]; cecropin P1 from *Sus scrofa* [14]; scorpine from this work; defensin from *Aeschna cyanea* [10] and a defensin from the scorpion *Leiurus quinquestriatus hebraeus* [11].

lower concentration than that of Shiva-3. The calculated ED_{50} were 10 μM for the gamete and 0.7 μM for the ookinete stages of development. These results indicate that scorpine could be a better candidate than Shiva-3, to be incorporated into the genome of genetically engineered malaria-resistant anophelines.

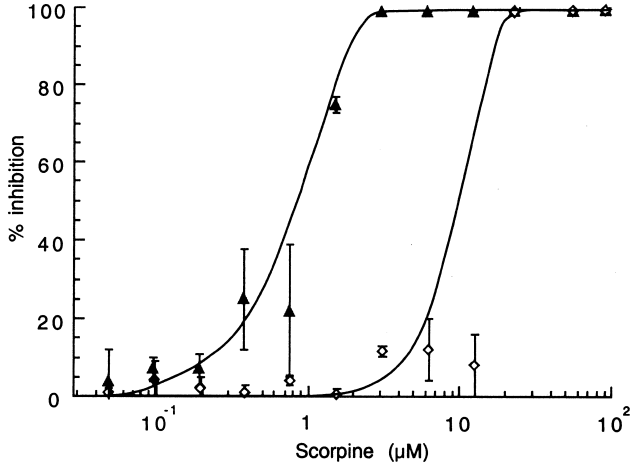

Fig. 3. Dose response curve of the effect of scorpine on the ookinete and fecundation (rosette) phases of the development of *P. berghei*. Inhibition of the ookinete development (closed triangles) and inhibition of rosette formation (open rhomboids) of *P. berghei* in vitro by the scorpine peptide. For the ookinete phase, at least 10000 red blood cells were counted, whereas for the rosette formation a minimum of 10 microscopic fields were directly examined, for each scorpine concentration.

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