

UNIVERSIDADE ESTADUAL DE CAMPINAS

Erika Maria Silva Freitas

ESTUDO DA INTERAÇÃO DA VERATRINA
COM O MÚSCULO ESQUELÉTICO POR
MÉTODOS HISTOQUÍMICOS,
ULTRAESTRUTURAIS E BIOQUÍMICOS

de cada uma das etapas da avaliação final
1. Assinatura do(a) candidato(a)
Erika Maria
Silva Freitas
e aprovada pela Comissão Julgadora.
07/11/2000
José Carlos Pires

Tese apresentada ao Instituto de
Biologia para obtenção do Título de
Mestre em Biologia Celular e Estrutural
na área de Biologia Celular

UNICAMP

BIBLIOTECA CENTRAL
SEÇÃO CIRCULANTE

Orientadora: Prof^a Dr^a Maria Alice da Cruz-Höfling

UNIDADE	B C
N.º CHAMADA:	1/UNICAMP
	F884e
V.	Ex.
TOMBO BC/	43760
PROC.	16-092107
C	☐
D	☒
PREC	R\$ 11,00
DATA	13/08/01
N.º CPD	

CM-00153948-3

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

F884e

Freitas, Erika Maria Silva

Estudo da interação da veratrina com o músculo esquelético por métodos histoquímicos, ultraestruturais e bioquímicos/Erika Maria Silva Freitas. - - Campinas-SP. [s.n.], 2000.
145f: illus.

Orientadora: Maria Alice da Cruz-Höfling
Dissertação (mestrado) – Universidade Estadual de Campinas.
Instituto de Biologia.

1. Músculo esquelético. 2. Alterações morfológicas. 3. Veratrina.
I. Cruz-Höfling, Maria Alice. II. Universidade Estadual de Campinas.
Instituto de Biologia. III. Título

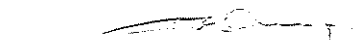
Data da defesa: 07/11/2000

BANCA EXAMINADORA

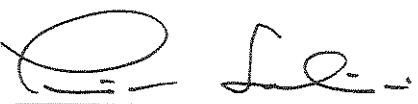
Profª Drª Maria Alice Cruz- Höfling (Orientadora)


(Assinatura)

Prof. Dr. Gerson Eduardo Rocha Campos


(Assinatura)

Profª Drª Tania de Fátima Salvini


(Assinatura)

Prof. Dr. Stephen Hyslop


(Assinatura)

UNICAMP
BIBLIOTECA CENTRAL
SEÇÃO CIRCULANTE

Dedico este Trabalho...

Aos meus pais e minha irmã que estiveram presentes em todos os momentos e permitiram a realização deste sonho.

Agradecimento Especial...

À Prof^a Dr^a Maria Alice da Cruz-Höfling pela orientação, dedicação dispensada e troca de experiências que enriqueceram minha vivência durante o meu curso de Mestrado.

Agradecimentos

Aos professores Dr. Gerson Eduardo Rocha Campos, Dr. Stephen Hyslop e Dr^a Tânia de Fátima Salvini pela honra de tê-los como membros da banca. Suas sugestões e críticas feitas durante a análise prévia da tese enriqueceram e contribuíram para a finalização deste trabalho;

À Prof^a Dr^a Maeli Dal Pai-Silva pela amizade, colaboração e por ter colocado a disposição toda a infra-estrutura necessária para desenvolver parte deste trabalho;

Ao Prof. Dr. Aníbal E. Vercesi pela colaboração e permissão concedida para que eu realizasse os ensaios bioquímicos no laboratório de Bioenergética;

Ao Prof. Dr. Arício X. Linhares pela disposição em auxiliar com análise estatística;

À Prof^a Dr^a Shirlei Recco Pimentel pelo acolhimento junto a esse curso de Pós-Graduação e por possibilitar a utilização do fotomicroscópio presente em seu laboratório;

Aos funcionários do laboratório de Microscopia Eletrônica – IB/Unicamp pelo auxílio na revelação dos negativos;

Ao Centro de Microscopia Eletrônica – IB/Unesp – Botucatu por permitir a análise do material ao microscópio eletrônico de transmissão e, principalmente a sra. Maria Helena Moreno, pelos auxílios prestados na preparação das amostras e na confecção das fotografias;

Ao Laboratório de Bioenergética, principalmente a Márcia Fagian pelo ensinamento e dedicação dispensada durante os ensaios bioquímicos;

Ao amigo Gustavo Henrique da Silva pelo inestimável auxílio durante os experimentos e confecção das fotografias;

À Sueli Michelin pela amizade e apoio técnico no desenvolvimento das técnicas histoenzimológicas;

À Djanira pelo ensinamento das técnicas cirúrgicas;

À Luciana e Gláucia pela amizade, carinho e receptividade junto ao departamento e por terem me socorrido durante os “momentos difíceis” na execução deste trabalho;

À Su, Vera, Selene, Luna e Verônica pela solidariedade nos momentos angustiantes e por compartilharem minhas vitórias conseguidas durante minha vida acadêmica e pessoal;

Aos funcionários dos Departamentos de Histologia & Embriologia e de Biologia Celular pela prestatividade e auxílio na execução de itens burocráticos;

À CAPES, FAEP-UNICAMP e FAPESP pelo suporte financeiro;

E a todos, não citados nominalmente aqui, que direta ou indiretamente, contribuíram para a execução deste trabalho.

Esta tese foi confeccionada em conformidade com o Regimento CCPG/001/98, parecer nº 1985/96 de janeiro de 1999 do Curso de Pós-Graduação em Biologia Celular e Estrutural. O formato adotado para a sua confecção foi o de apresentá-la sob forma de artigos já em língua inglesa, prontos para serem submetidos à publicação, de acordo com os Artigos 1 e 2 do referido regimento.

SUMÁRIO

Resumo	10
Abstract	13
1. Introdução	15
1.1. A veratrina	15
1.2. Considerações Gerais sobre o Músculo Estriado Esquelético	21
1.2.1. Retículo Sarcoplasmático e os Túbulos T	23
1.2.2. Mitocôndrias	24
1.3. Tipos de Fibras Musculares	25
1.4. Canais de Sódio	29
1.5. Canais de Sódio e Fibras Musculares	31
1.6. Toxinas que atuam em Canais de Sódio	32
1.6.1. Toxinas de Sítio I	33
1.6.2. Toxinas de Sítio II	35
1.6.3. Toxinas de Sítio III	36
1.6.4. Toxinas de Sítio IV	38
1.6.5. Toxinas de Sítio V	39
1.6.6. Outras Toxinas que atuam em Canais de Sódio	39
1.7. Toxinas Ativadoras de Canais de Sódio e Danos Neuromusculares	40
1.8. Objetivos	42
2. Artigos	
Resultados: Organização	45
2.1 Artigo 1	47
2.2 Artigo 2	69
2.3 Artigo 3	89
2.4 Artigo 4	113
3. Conclusões	133
4. Referências Bibliográficas	134

RESUMO

A veratrina é o extrato comercial obtido do *Schoenocaulon officinale* (Liliaceae), planta nativa da América do Sul e Central, que ativa os canais de sódio-voltagem dependentes, induzindo despolarização persistente e aumento da permeabilidade aos íons sódio em fibras musculares ou nervosas. O objetivo do presente trabalho foi investigar os efeitos da veratrina sobre o músculo esquelético de camundongos e peixes. A expressão fenotípica das fibras oxidativas, oxidativa-glicolíticas e glicolíticas no músculo sóleo (de contração lenta, com fibras predominantemente oxidativas) e EDL (de contração rápida, com maioria de fibras glicolíticas) de camundongos, e do músculo lateral de tilápia foi examinada em vários intervalos de tempo após injeção intramuscular da veratrina. As alterações ultraestruturais nos músculos foram analisadas ao microscópio eletrônico de transmissão. Os efeitos da veratrina foram também examinados em mitocôndrias isoladas de músculo esquelético de camundongos e ratos, e em mitocôndrias de fígado de ratos.

Nos camundongos, as fibras oxidativas e as fibras oxidativa-glicolíticas, ambas ricas em mitocôndrias, mostraram um decréscimo da atividade das enzimas oxidativas (NADH-TR) após a veratrina. As fibras glicolíticas, com menor número de mitocôndrias foram menos sensíveis ao alcalóide. Já nas tilápias, foram as fibras oxidativas e as glicolíticas que apresentaram maior susceptibilidade ao dano metabólico sob ação do alcalóide.

Com relação à atividade contrátil houve um significativo decréscimo ($P=0.001$) no número de fibras tipo IIB e um aumento no número de fibras tipo I de camundongos após injeção do alcalóide no EDL. Ao contrário, no músculo sóleo houve um decréscimo nas fibras tipo I e IIA. Além disso, houve o aparecimento de fibras indiferenciadas nos dois

músculos ao longo dos 60 min do tratamento. Em contraposição, não houve alterações na atividade contrátil dos três tipos de fibras presentes no músculo lateral das tilápias ao longo dos 30 min após a administração do alcalóide, exceto pelo aparecimento de fibras indiferenciadas aos 60 min.

As alterações mionecróticas produzidas pela veratrina incluíram dilatação das cisternas terminais do retículo sarcoplasmático, hipercontração de miofibrilas, desorganização de sarcômeros, dissociação e/ou ruptura de miofilamentos e dano mitocondrial. Estas alterações se diferenciaram quanto à gravidade e ao tempo de aparecimento nos diferentes músculos. Por exemplo, a dilatação das cisternas do retículo sarcoplasmático ocorreu mais precocemente e foi mais evidenciada nas fibras musculares do EDL do que no sóleo, ao passo que foram sutis nas fibras das tilápias. As alterações mitocondriais entretanto, foram mais precoces e proeminentes no sóleo do que no EDL.

Os efeitos da veratrina nas mitocôndrias isoladas mostraram que o alcalóide desacopla a oxidação fosforilativa em músculos esqueléticos de camundongos e ratos, devido a um aumento na transição da permeabilidade da membrana interna a prótons, de modo não específico. Diferentemente, a atividade respiratória nas mitocôndrias isoladas de fígado foi inibida após a administração da veratrina.

Nós concluímos que a interação da veratrina com as fibras musculares é espécie-específica, músculo-específica e tipo de fibra específica, e que a interação da veratrina com as mitocôndrias é tecido(órgão)-específica. As alterações mionecróticas induzidas pela veratrina foram atribuídas ao maior influxo de íons sódio e de água para o interior das células, já que estes efeitos foram antagonizados pela tetrodotoxina, um bloqueador de canais de sódio. Entretanto, não podemos descartar a possibilidade de que outros mecanismos, tais como a mobilização de Ca^{2+} das cisternas do retículo sarcoplasmático ou

de mitocôndrias e/ou a penetração da toxina para o interior das células possam estar envolvidos.

ABSTRACT

Veratrine, a commercial extract from the South and Central America plant *Schoenocaulon officinale* (Liliaceae), activates voltage-dependent Na^+ channels to produce persistent depolarization and an increase in sodium ion permeability in muscle fibers or nerves. The aim of this study was to investigate the effects of the veratrine alkaloid on skeletal muscle of mice and fish. The phenotypic expression of oxidative, oxidative-glycolytic and glycolytic fibers in soleus muscle (slow-twitch, with predominantly oxidative fibers) and EDL muscle (fast-twitch, with predominantly glycolytic fibers) of mice, and lateral muscle of tilapia were examined at various times after intramuscular injection of veratrine. The ultrastructural changes in the muscles were analyzed by transmission electron microscopy. The veratrine effects were also examined in isolated mitochondria from mice and rats skeletal muscle, and in isolated rat liver mitochondria.

In mice, oxidative and oxidative-glycolytic fibers, which are rich in mitochondria, showed decreased oxidative enzyme activity (NADH-TR reaction) after veratrine. Glycolytic fibers, which have fewer number of mitochondria, were less sensitive to the alkaloid. However, the oxidative and glycolytic fibers of tilapia were more susceptible to damage by veratrine.

There was a significant decrease ($P=0.001$) in the number of type IIB fibers and an increase in the number of type I fibers in EDL muscle of mice (m-ATPase reaction) after alkaloid injection. In contrast, there was a decrease in type I and IIA fibers of the *soleus* muscle. Moreover, undifferentiated fibers were present in both muscles throughout the 60 min treatment. Contrarily, there were not alterations in the contractile activity of the three

types of fibers present in tilapia lateral muscle up to 30 min after alkaloid injection, but undifferentiated fibers were seen after 60 minutes.

The myonecrotic alterations produced by veratrine included swelling of the terminal cisternae sarcoplasmic reticulum, hypercontraction of myofibrils, disorganization of sarcomeres, dissociation and/or rupture of myofilaments and mitochondrial damage. These alterations differed in gravity and duration in different muscles. Thus, swelling of the sarcoplasmic reticulum cisternae was earlier and more prominent in EDL muscle fibers than in the *soleus*, and was subtle in tilapia muscle fibers. The mitochondrial alterations though were earlier and more prominent in the *soleus* than in the EDL.

The effects of veratrine in isolated mitochondria showed that the alkaloid uncouples oxidative phosphorylation in mice and rats skeletal muscle due to an increase in unspecific permeability transition of the inner membrane to protons. Differently, the respiratory activity on isolated liver mitochondria was inhibited after veratrine administration.

We conclude that the interaction of veratrine with muscle fibers is species-specific, muscle-specific and fiber type-specific, and that the interaction of the veratrine with mitochondrial is tissue(organ)-specific. The veratrine induced-myonecrotic alterations were attributed to the higher influx of Na^+ ions and water into cells, since they were antagonized by tetrodotoxin, a Na^+ channel blocker. However, the involvement of other mechanisms, such as the mobilization of calcium ions from sarcoplasmic reticulum cisternae or mitochondria, and/or the entry of toxin into cells cannot be ruled out.

1. INTRODUÇÃO

A introdução foi organizada de forma a colocar pontualmente informações consideradas relevantes como substrato teórico para as exposições subseqüentes contidas nos artigos originados do projeto experimental. Assim, discorreremos inicialmente no item 1.1 sobre a veratrina no que respeita à origem, estrutura química, efeitos clínicos, estudos experimentais, aspectos farmacológicos e mecanismos de ação. A seguir serão tecidas algumas considerações gerais sobre as características morfofuncionais do tecido muscular que fazem das células desse tipo altamente especializado de tecido, aptas a serem excitadas por substâncias químicas específicas (item 1.2). Também serão abordados aspectos relativos aos diferentes tipos de fibras musculares já que será investigada uma possível "preferência de ação da veratrina" a um tipo particular de fibra muscular (item 1.3). Rapidamente abordaremos aspectos morfofuncionais dos modelos de canais de sódio e daremos uma idéia das toxinas integrantes de venenos ou substâncias tóxicas de organismos e suas ações nas fibras musculares esqueléticas (item 1.4-1.7).

1.1. A veratrina

Os alcalóides de plantas do gênero *Veratrum* são extraídos das sementes ou rizomas das plantas da subordem Melanthaceae pertencentes à família Liliaceae. As espécies mais importantes onde estes alcalóides foram isolados são *Veratrum album* (Figura 1), encontrada na Europa e norte da Ásia, *Veratrum viride*, encontrada na América do Norte, e *Schoenocaulon officinale*, encontrada principalmente no sul da América do Norte, América do Sul (Venezuela) e Central (Guatemala) (Benforado, 1967; Catterall, 1980; Honerjäger, 1982; Ulbricht, 1998). As sementes de "sabadilla" (nome popular do *S. officinale*) foram inicialmente utilizadas como inseticidas pelas civilizações pré-colombianas dos astecas, maias e incas (Crosby, 1971).

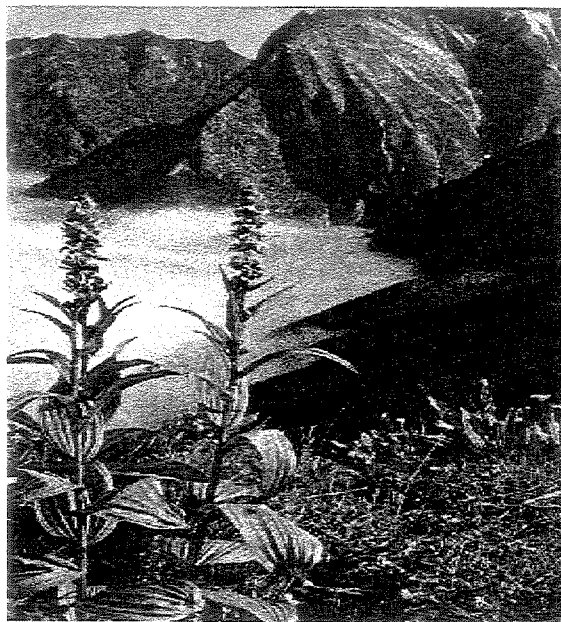


Figura 1: Exemplar de *Veratrum album* (ilustração retirada do livro *Fleurs des Alpes*- Paula Kohlhaupt)

A veratrina é o nome dado ao extrato comercial obtido de *S. officinale*. Contém principalmente uma mistura de dois ésteres alcalóides, a cevadina e a veratridina, sendo este último a toxina mais potente dos alcalóides do gênero *Veratrum* (Benforado, 1967; Catterall, 1980). Estes alcalóides são compostos policíclicos, lipossolúveis, com estrutura química esteróide contendo um átomo de nitrogênio secundário ou terciário dentro do anel (Figura 2) (Benforado, 1967; Catterall, 1980).

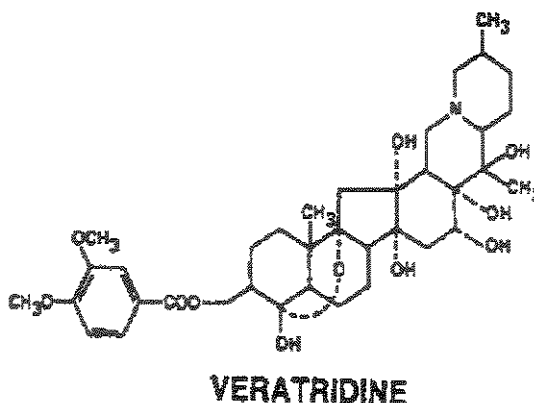


Figura 2: Representação da estrutura química da veratridina (Catterall, 1980).

Apesar da purificação dos alcalóides do gênero *Veratrum* ter se iniciado na década de 40 e os primeiros ensaios farmacológicos terem sido feitos nessa época (Kraye & Acheson, 1946), apenas em 1970 a veratridina isolada passou a ser utilizada comercialmente (Ulbricht, 1998).

A utilização dessas plantas com propósitos medicinais remonta a períodos bem anteriores. O primeiro relato da utilização clínica dos extratos brutos de plantas do gênero *Veratrum* ocorreu em 1867, em pacientes com hipertensão arterial (Benforado, 1967; Catterall, 1980). Em 1939, Jarisch baseando-se nos estudos de von Bezold, observou efeitos cardiovasculares, como hipotensão e bradicardia, após a administração de pequenas doses intravenosas de veratrina em animais e humanos. Estes efeitos não envolviam apenas ações cardiovasculares. Os pulmões, o seio carotídeo e as artérias carótidas também apresentavam importantes áreas receptoras para estes alcalóides (para revisão ver Benforado, 1967).

Os efeitos cardiovasculares provocados pela veratrina foram denominados de “efeitos Bezold-Jarisch”, termo sugerido por Kraye em 1953 (*cf.* Benforado, 1967). Aliado ao efeito hipotensivo, a veratrina produzia também outros efeitos colaterais indesejáveis.

Os graves efeitos colaterais advindos do uso destes alcalóides, foram paralisia da face e tórax, arritmias cardíacas e miotonia, o que levou à restrição do uso dessa substância como ferramenta terapêutica. Estudos têm demonstrado que os alcalóides do *Veratrum*,

quando são administrados por via intravenosa em doses de $\mu\text{g/kg}$ do animal, afetam os sistemas respiratório, cardiovascular, nervoso e muscular (Benforado, 1967).

Um dos primeiros ensaios fisiológicos com a veratridina foi realizado por Ulbricht (1965; 1969) que observou a ação deste alcalóide sobre os nodos de Ranvier de fibras nervosas de rã utilizando a técnica de "voltage-clamp". É interessante notar que as membranas excitáveis das fibras nervosas mielínicas têm nos nodos de Ranvier o local de maior concentração de canais de sódio, os quais são responsáveis pela condução saltatória do impulso elétrico (Ritchie & Rogart, 1977; Ritchie & Chiu, 1981).

Estudos posteriores vieram demonstrar que estes alcalóides alteram a corrente de sódio sensível à tetrodotoxina (Horackova & Vassort, 1973), causando ativação persistente dos canais de sódio (Catterall, 1980; Honerjäger *et al.*, 1992). Os alcalóides do *Veratrum* induzem despolarização e respostas elétricas iterativas em membranas excitáveis de fibras musculares ou nervosas (Macfarlane & Meares, 1958; Ulbricht, 1969), denominadas de "resposta veratrínica", ou seja, um único estímulo aplicado em membrana de célula excitável provoca descarga de uma série de potenciais de ação (Benforado, 1967).

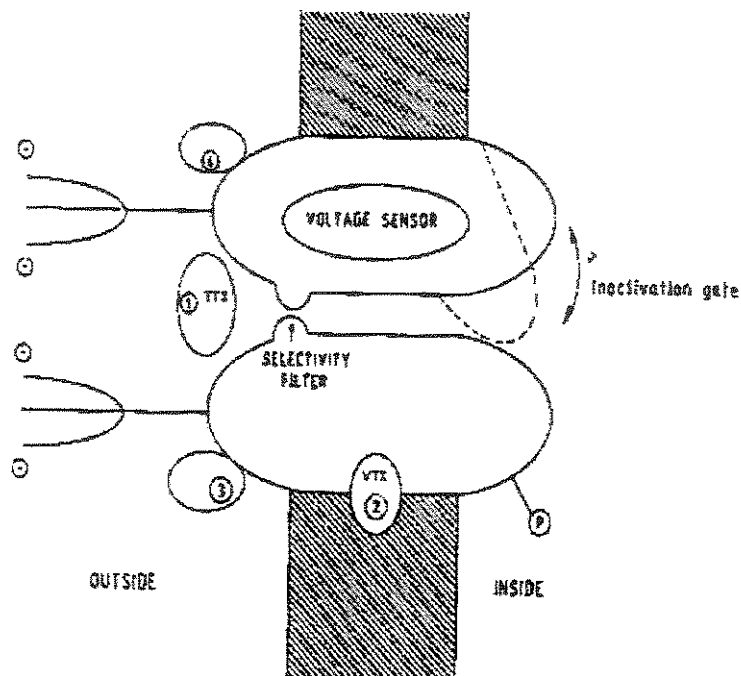


Figura 3: Representação dos sítios de ação da veratrina (VTX) e da tetrodotoxina (TTX) - esquema modificado de Becker & Gordon (1992)

Foi demonstrado que a veratrina liga-se, de modo reversível, ao sítio 2 dos canais de sódio (Figura 3), caracterizado como sítio hidrofóbico responsável pela ativação do canal (Becker & Gordon, 1992). O aumento da permeabilidade ao íon Na^+ pode ser inibido pela tetrodotoxina (TTX), um composto guanidínico heterocíclico que bloqueia esses canais iônicos (Catterall, 1980).

Durante a década de 80, uma série de pesquisas esteve voltada para a elucidação das ações dos alcalóides pertencentes ao gênero *Veratrum*, principalmente focalizando seus efeitos sobre os canais de sódio presentes nas membranas de fibras nervosas mielínicas e musculares esqueléticas. Assim, em 1985, Vital Brazil & Fontana investigaram o efeito *in vitro* produzido pela veratrina. Os autores usaram várias doses do alcalóide (0.4 , 2.0 e 10.0 $\mu\text{g/ml}$) em cinco diferentes regiões da preparação de nervo frênico-diafragma de ratos, previamente "mapeadas" pelos autores utilizando o critério de proximidade à área de inervação motora. Os autores observaram que a despolarização nas distintas regiões do músculo diafragma causada pelo alcalóide era dependente da dose. Na concentração de 0.4 $\mu\text{g/ml}$, a veratrina despolarizava as regiões 1 e 2, e na de 2.0 $\mu\text{g/ml}$, as regiões 1, 2 e 3, mais próximas à placa motora. Com a concentração de 10 $\mu\text{g/ml}$ de veratrina, a despolarização ocorria em todas as cinco regiões selecionadas pelos autores. Concluíram que esta despolarização seletiva de regiões do músculo poderia ser causada por uma distribuição diferenciada de canais de sódio ativados pela veratrina ou pela existência de quantidades diferentes de tipos de canais de sódio em áreas distintas do músculo. Já está estabelecido que existe maior concentração de canais de sódio nas regiões próximas de junções neuromusculares (Caldwell *et al.*, 1986). Num trabalho posterior, Vital Brazil & Fontana (1993) demonstraram que a veratrina e outras toxinas ativadoras dos canais de sódio tais como a crotamina, o veneno bruto da aranha *Phoneutria nigriventer* e sua fração tóxica PhTx2, produziam maior despolarização no sarcolema das regiões juncionais do que das regiões extrajuncionais. Entretanto, a veratridina e a batrachotoxina, alcalóide extraído da pele de rãs pertencentes ao gênero *Phyllobates*, também toxinas ativadoras dos canais de sódio, despolarizaram uniformemente o sarcolema, não ocorrendo predisposição por uma região ou outra. Uma possibilidade encontrada pelos autores para explicar essa diferença de despolarização seria a existência de um subtipo de canal de sódio encontrado em maior

número nas regiões juncionais, sensível preferencialmente à veratrina, à crotamina, ao veneno bruto da *P. nigriventer* e sua fração tóxica PhTx2. Já a veratridina e a batrachotoxina interagiriam com um subtipo de canal de sódio distribuído uniformemente no sarcolema ou ativariam canais de sódio "silenciosos" presentes nas regiões extrajuncionais, isto é, canais de Na^+ -voltagem insensíveis, responsáveis pela despolarização uniforme da membrana da fibra muscular (Vital Brazil & Fontana, 1993).

Os alcalóides pertencentes ao gênero *Veratrum* podem também agir nos canais de sódio presentes nas membranas dos músculos esqueléticos de invertebrados. Lehouelleur e colaboradores (1988), utilizando preparações de músculo flexor abdominal do pitu ou camarão d'água doce *Procambarus clarkii*, notaram que após a aplicação dos alcalóides veratrina ou veratridina *in vitro*, também ocorria despolarização das membranas excitáveis, provocando descargas de potenciais de ação. Estes potenciais eram Na^+ -dependentes, Ca^{2+} -dependentes e TTX-resistentes.

A ativação dos canais de sódio induzidos por esses alcalóides é também dependente da dose e concentração. Em um estudo experimental *in vitro* com veratridina, foram utilizadas fibras isoladas do músculo semitendinoso de rãs adultas *Rana catesbiana*. A incubação com veratridina (1 μM) mostrou que nesta concentração, a toxina não afeta as correntes de íons Na^+ e Ca^{2+} (Sutro, 1986). Em 1994, Nánási e colaboradores utilizando fibras isoladas do músculo semitendinoso de rã *Rana pipiens*, mostraram que a veratridina em altas concentrações (100 μM) ativava esses canais iônicos.

Benders e colaboradores (1997) demonstraram que a veratridina em cultura de células musculares esqueléticas humanas, obtidas dos músculos quadríceps, bíceps e reto abdominal, causava despolarização do sarcolema e ativação do complexo DHPR-RyR (dihidropiridina-rianodina), receptores encontrados em túbulos-T e retículo sarcoplasmático. Através da ativação desse complexo, ocorria a liberação de Ca^{2+} do retículo sarcoplasmático. O uso prévio de TTX inibia parcialmente a liberação de íons Ca^{2+} do retículo sarcoplasmático, isto devido à coexistência de receptores TTX-sensível e TTX-resistente ligados aos canais de Na^+ .

Há mais de um século realizam-se ensaios farmacológicos e fisiológicos com os alcalóides do *Veratrum*. Por constituírem uma classe de toxinas que ativa os canais de sódio

têm sido eleitos como ferramentas no estudo dos mecanismos de funcionamento desses canais iônicos. Por outro lado, estudos direcionados para ampliar o conhecimento sobre os sítios-alvos de ação da veratrina são restritos, principalmente no que diz respeito à ação no tecido muscular esquelético, *in vivo*.

1.2. Considerações gerais sobre o músculo estriado esquelético

O músculo esquelético é constituído por células ou fibras orientadas paralelamente, circundadas por tecido conjuntivo. São células alongadas e multinucleadas, com núcleos localizados periféricamente, podendo atingir mais de 30 cm de comprimento e diâmetro de 10-100 μm (Dubowitz, 1985). Entretanto, o comprimento da fibra muscular pode variar de acordo com o sexo (Brooke & Engel, 1969a), com a idade do indivíduo (Brooke & Engel, 1969b), com o músculo estudado (Brownlow *et al.*, 1989) e com a atividade física e alimentação (Mahon *et al.*, 1984).

As fibras musculares são inervadas por fibras nervosas motoras que formam estruturas denominadas placas motoras ou junção neuromuscular (Ross & Rowell, 1993).

As miofibrilas ocupam cerca de 80-90% do volume de uma fibra muscular, dependendo do tipo de fibra. São compostas por uma seqüência de unidades contráteis repetitivas denominadas de sarcômeros (Harris & Cullen, 1990).

O sarcômero é definido como uma unidade morfofuncional que se estende de uma linha Z à outra. A organização do sarcômero é mantida pela presença das proteínas titina, responsável pela elasticidade do tecido muscular estriado (Linke *et al.*, 1996) e nebulina, importante na regulação do comprimento dos filamentos de actina durante o desenvolvimento do músculo (Alberts, 1994a). Visto ao microscópio eletrônico, o sarcômero é constituído por bandas A (escuras) ao centro, alternadas por bandas I (claras). As bandas I são bissectadas por uma linha densa denominada de linha Z (Harris & Cullen, 1990).

As bandas I (isotrópicas sob luz polarizada) são compostas exclusivamente pelos miofilamentos finos contendo actina, troponina e tropomiosina. As bandas A (anisotrópicas) são compostas por parte dos miofilamentos finos e pelo comprimento total

dos miofilamentos grossos, tendo centralmente a banda H, que corresponde à região da miofibrila onde é encontrada somente a miosina, sem interposição dos miofilamentos finos de actina. A linha M aparece no centro de cada banda H, e é constituída pelas projeções transversais do centro do filamento de miosina. Neste local encontra-se também a creatinofosfoquinase (CK), enzima importante para o metabolismo energético da miofibrila. A linha Z é uma estrutura altamente flexível, capaz de suportar o deslizamento dos miofilamentos durante os processos de contração e relaxamento muscular (Harris & Cullen, 1990; Esbérard, 1991). Duas importantes proteínas estão associadas à linha Z: α -actinina, proteína pertencente à família das espectrinas, responsável por promover a ligação cruzada das actinas (Alberts, 1994a), e a desmina, responsável pela ligação entre linhas Z adjacentes e/ou ligação das linhas Z com a membrana plasmática (Li *et al.*, 1997; Capetanaki *et al.*, 1997).

Estudos feitos com difração de raios-X e microscopia eletrônica vêm contribuindo na definição do arranjo estrutural da fibra muscular, e concomitantemente na elucidação dos eventos moleculares envolvidos no processo da contração muscular. Através dessas técnicas chegou-se à conclusão de que a molécula de miosina apresenta um arranjo estrutural em forma de bastão com 20 nm de comprimento e 2-3 nm de diâmetro, disposto em dupla α -hélice. A miosina pode dissociar-se em duas cadeias pesadas idênticas e dois pares de cadeias leves. A porção caudal da miosina é chamada de meromiosina leve e a porção que contém as cabeças, de meromiosina pesada. As cabeças das moléculas de miosina apresentam atividade ATPásica, responsável por liberar energia para o processo de contração muscular (Ésberard, 1991; Squire, 1997).

O filamento de actina é constituído por monômeros de actina G (globular) que polimerizados formam duas cadeias torcidas em α -hélice e constituem a actina F (filamentar). O arranjo atômico da actina G foi determinado apenas em 1990 por Kabsch e colaboradores (*cf.* Squire, 1997). O monômero de actina tem quatro subdomínios: 1 (16400 Da); 2 (4000 Da); 3 (11500 Da) e 4 (10000 Da). Os subdomínios 3 e 4 estão localizados no lado interno dos filamentos finos, interagindo principalmente com os subdomínios 3 e 4 de outros monômeros de actina, enquanto o subdomínio 1 possui o sítio de ligação à miosina. Os filamentos de actina possuem também as "proteínas regulatórias" tropomiosina,

troponina e nebulina. As moléculas de tropomiosina são formadas por duas cadeias de polipeptídeos, cada uma com estrutura α -helicoidal que se associam à actina F e ocupam o sítio entre as actinas G seis e sete. Cada tropomiosina está associada com um complexo globular de troponina (80.000 Da) que compreende três subunidades: troponina C (18.000 Da), sítio de ligação a íons Ca^{2+} ; troponina I (21.000 Da), sítio inibitório do acoplamento actina-miosina e troponina T (30.500 Da), sítio de ligação a tropomiosina (*cf.* Squire, 1997).

A contração muscular inicia-se pela combinação de íons cálcio com a subunidade Tn-C da troponina e o deslocamento da tropomiosina, conseqüentemente expondo os sítios fixadores para a cabeça da molécula de miosina presentes no filamento de actina. Em seguida, a miosina catalisa a hidrólise do ATP (adenosina trifosfato) em ADP (adenosina difosfato) e fosfato inorgânico, promovendo o deslizamento e a sobreposição dos miofilamentos e, concomitantemente o encurtamento da fibra muscular (Esbérard, 1991).

1.2.1. Retículo Sarcoplasmático e Túbulos-T

O retículo sarcoplasmático apresenta-se nas fibras musculares como uma rede de cisternas do retículo endoplasmático liso que envolve as miofibrilas. É composto por compartimentos distintos responsáveis pela regulação de íons cálcio no interior das células. Divide-se em dois compartimentos, retículo sarcoplasmático longitudinal e cisternas terminais.

O retículo sarcoplasmático longitudinal apresenta alta concentração de Ca^{2+} -ATPase em sua membrana e é responsável pela recaptação de íons cálcio para o interior do retículo, interrompendo a atividade contrátil da fibra muscular. As cisternas terminais são responsáveis pelo armazenamento e liberação de cálcio do retículo sarcoplasmático. Apresentam a calsequestrina, uma proteína de ligação ao cálcio, e o receptor rianodina, responsável pela liberação de íons cálcio do interior do retículo (Flucher, 1992). Após a liberação dos íons cálcio as proteínas contráteis são ativadas e segue-se a contração muscular.

Os túbulos-T ou sistema-T é uma rede complexa de invaginações tubulares da membrana celular ou sarcolema responsável pela propagação de potenciais de ação para o interior da fibra muscular de forma a atingir instantaneamente as miofibrilas (Flucher, 1992). Há maior concentração de túbulos-T nas fibras musculares de contração rápida do que nas de contração lenta (Eastwood *et al.*, 1982). Os túbulos-T apresentam os canais de Na^+ voltagem-dependentes, a bomba Na^+/K^+ -ATPase e altas concentrações dos receptores 1,4-dihidropiridina (DHPs) em suas membranas. O DHP é um complexo de multisubunidades pertencente à classe de canais de cálcio tipo-L, que age como um sensor de voltagem no mecanismo de excitação-contração muscular (Roseblatt *et al.*, 1981; Flucher, 1992). Em cada lado do túbulo-T existem cisternas terminais do retículo sarcoplasmático formando um complexo especializado denominado de tríade. A localização das tríades nas fibras musculares difere de acordo com as espécies animais. Por exemplo, nos peixes teleósteos as tríades se localizam preferencialmente ao longo da linha Z e nos mamíferos, na junção das bandas A e I (*cf.* Ogata, 1988). Esta disposição das tríades é estratégica para que a liberação de íons cálcio das cisternas terminais do retículo sarcoplasmático ocorra nos locais onde há sobreposição dos miofilamentos (Esbérard, 1991).

1.2.2. Mitocôndrias

No espaço intermiofibrilar, juntamente com os perfis das cisternas do retículo sarcoplasmático e dos túbulos-T, alinham-se as mitocôndrias, organelas responsáveis pela geração de energia para a fibra muscular e pela regulação, em conjunto com o retículo sarcoplasmático, da concentração intracelular de íons cálcio. Além dessa localização, as mitocôndrias podem se acumular nas regiões subsarcolemas ou no sarcoplasma subsináptico.

Ultraestruturalmente, as mitocôndrias são esféricas ou ovóides e a disposição e o número de mitocôndrias são variáveis nos diferentes tipos de fibras musculares existentes (*cf.* Ogata, 1988).

1.3. Tipos de fibras musculares

Nem todas as fibras musculares são fisiológica, bioquímica e metabolicamente iguais. As diferenças entre esses aspectos da fibra muscular são dependentes do tipo de terminal nervoso, e controlado geneticamente. As fibras musculares, ainda na fase de miotubos, dependem da inervação para sofrerem o processo de diferenciação (Jansen & Fladby, 1990). Uma série de experimentos tem mostrado a existência de uma interação interdependente entre células musculares e a sua inervação motora. Por outro lado, fatores tróficos, que são permutados entre terminais nervosos e fibras musculares, estão envolvidos tanto na determinação do tipo de fibra muscular, como na reinervação das fibras musculares, quando o neurônio motor ou as fibras nervosas motoras são afetados (Seckel, 1990).

A distribuição dos tipos de fibras musculares é variável nas espécies de animais. Esta heterogeneidade na proporção de tipos de fibras musculares é devido a um alto grau de especialização funcional dos diferentes músculos (Pette & Staron, 1990).

Em 1873, Ranvier diferenciou dois tipos de fibras constituintes do tecido muscular esquelético: fibras vermelhas e fibras brancas (para revisão ver Pette & Staron, 1990). As fibras vermelhas são ricas em citocromo e mioglobina, dando a coloração avermelhada a fresco ao tecido muscular. Já as fibras brancas são pobres em citocromo e mioglobina (Ogata, 1958), sendo esbranquiçadas a fresco.

Com o aperfeiçoamento dos métodos histológicos, fisiológicos e histoquímicos de análise dos tipos de fibras musculares, também foi sendo implementada a classificação dos diferentes tipos de fibras musculares (Pette & Staron, 1990). A identificação dos diferentes tipos de fibras pode ser realizada utilizando-se várias colorações e reações histoquímicas, dentre as quais destacam-se as reações do ácido periódico de Schiff (PAS), nicotinamida adenina dinucleotídeo hidrogênio-tetrazólio redutase (NADH-TR), succinato desidrogenase (SDH) e adenina trifosfatase miofibrilar (ATPase-m).

A reação de PAS é utilizada para quantificar a concentração de glicogênio nas fibras musculares (Peter *et al.*, 1972). Desta maneira, as fibras brancas que possuem grande estoque de glicogênio em seu citoplasma apresentam positividade mais intensa à reação quando comparadas às fibras vermelhas.

As reações histoquímicas NADH-TR e SDH revelam a atividade metabólica de uma fibra muscular através da redução de elementos da cadeia respiratória (Ogata & Mori, 1964; Peter *et al.*, 1972; Dubowitz, 1985). A NADH-TR é uma diaforase que reduz os sais de tetrazólio utilizando a coenzima NADH. Os elétrons do NADH entram na cadeia respiratória através da NADH-Q redutase (ou NADH desidrogenase ou Complexo I). A etapa inicial do processo é a ligação de NADH e a transferência de seus dois elétrons para a flavina mononucleotídeo (FMN), grupamento prostético desse complexo para originar a forma reduzida FMNH₂: $\text{NADH} \rightarrow \text{NAD}^+ + \text{H}^+ + 2 \text{e}^-$



Os elétrons são então transferidos do FMNH₂ para uma série de aglomerado ferro-enxofre (Fe-S). Por último, os elétrons nos aglomerados Fe-S de NADH-Q são encaminhados à coenzima Q, também conhecida como ubiquinona. A coenzima Q é reduzida a um radical livre anionte semiquinona pela captação de um elétron. A redução desse intermediário ligado à enzima por um segundo elétron, origina finalmente o ubiquinol (QH₂) (*cf.* Stryer, 1996).

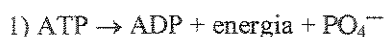
Na etapa da oxidação do ferro, o elétron é captado pelos sais de tetrazólio (NBT-Nitroblue Tetrazolium) formando um precipitado, visto na microscopia de luz como uma coloração azulada.

A SDH é uma enzima oxidativa intra-mitocondrial que participa do ciclo de Krebs e sua detecção histoquímica é um índice para a atividade deste ciclo. Age sobre um substrato específico transferindo hidrogênio do substrato diretamente para uma aceptor. O princípio da transferência de elétrons para os sais de tetrazólio é o mesmo descrito para a reação do NADH-TR (Nachlas *et al.*, 1957).

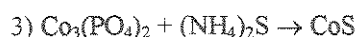
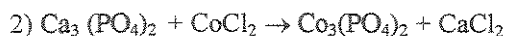
As fibras vermelhas, que possuem maior concentração de mitocôndrias, quando comparadas a outros tipos de fibras apresentam muitos depósitos de sais de formazana no interior da célula, caracterizando seu metabolismo oxidativo. As fibras brancas apresentam menor concentração desses sais, caracterizando seu metabolismo glicolítico. Existem fibras musculares que apresentam características intermediárias quanto à atividade metabólica, sendo chamadas de fibras oxidativa-glicolíticas (intermediárias).

A reação histoquímica ATPase-m (Brooke & Kaiser, 1970) é utilizada para avaliar as características contráteis das fibras musculares e diferenciá-las em três tipos principais denominados de I, IIA e IIB (Peter *et al.*, 1972; Dubowitz, 1985). A ATPase-m é uma fosfatase responsável pela hidrólise do ATP (adenosina trifosfato) em ADP (adenosina difosfato) e Pi (fosfato inorgânico). Na fibra muscular, a saída de íons cálcio do retículo sarcoplasmático em resposta ao estímulo nervoso ativa a atividade ATPásica (presente na cabeça da miosina). A ATPase separa o fosfato do ATP que se liga ao cálcio da solução, formando o fosfato de cálcio. O fosfato do cálcio é insolúvel e precipita-se no local da reação. Para que o produto da reação torne-se visível, o fosfato de cálcio é tratado com cloreto de cobalto. O cálcio é trocado pelo cobalto, formando o fosfato de cobalto que reage com sulfeto de amônio, formando o sulfeto de cobalto que apresenta a coloração marrom ou preta. Utiliza-se na reação um meio de incubação em pH 9,4, precedido de pré-incubação em meio ácido (pH 4,3-4,8) ou alcalino (pH 10,2-10,6).

Etapas da reação de ATPase-m:



proporcional a enzima ATPase



precipitado visto na microscopia de luz

A análise da atividade ATPase-m após pré-incubação em diferentes pHs mostra que os tipos de fibras estão relacionados com a distribuição das isoformas da cadeia pesada de miosina (Staron & Pette, 1986). Assim, para a identificação das fibras musculares, também podem ser utilizadas reações imunohistoquímicas, utilizando-se anticorpos específicos para a miosina (Pierobon-Bormioli *et al.*, 1981; Gorza, 1990). Através das técnicas de imunohistoquímica, nota-se a presença de quatro tipos de fibras musculares classificadas como tipos I, IIA, IIB e IIC. Entretanto, o tipo IIC só foi encontrado em fibras regenerativas contendo a isoforma de cadeia pesada de miosina embrionária e neonatal (Schiaffino *et al.*,

1986), capaz de se diferenciar em tipo IIA ou IIB (Dubowitz, 1985). Um outro tipo de fibra muscular denominado de IIX/IIID foi encontrado em músculos normais de ratos, camundongos e cobaias (Gorza, 1990). É um tipo intermediário que surge no processo de conversão do tipo IIB para o tipo IIA ou vice-versa (Zardini & Parry, 1994).

A inter-relação da atividade metabólica com a contrátil sugeriu uma nova terminologia para os três tipos principais de fibras musculares: fibras oxidativas e de contração lenta ("slow twitch oxidative"- SO); fibras glicolíticas e de contração rápida ("fast twitch glycolytic"- FG) e fibras com características intermediárias quanto ao metabolismo e de contração rápida ("fast twitch oxidative glycolytic"- FOG) (Peter *et al.*, 1972; Dubowitz, 1985; Ogata, 1988). Entretanto, alguns autores discordam desta classificação, pois a associação dos métodos para avaliar a atividade das enzimas oxidativas e a atividade ATPásica, pode conduzir a interpretações errôneas de tipagem de fibras (*cf.* Pette & Staron, 1997).

No caso da maioria das espécies de peixes, os tipos de fibras musculares apresentam-se distribuídos da seguinte maneira: fibras musculares oxidativas ou tipo I podendo aparecer na região subdermal como uma camada uniforme ao longo de todo o corpo (Egginton & Johnston, 1982; Hoyle *et al.*, 1986), ou aparecer numa configuração triangular somente na região da linha lateral (Walesby & Johnston, 1980; Te Kronie *et al.*, 1983; Hoyle *et al.*, 1986); fibras musculares glicolíticas ou tipo IIB aparecem constituindo a maior parte da massa corporal (Hoyle *et al.*, 1986; Weartherley & Gill, 1987) e fibras musculares oxidativa-glicolíticas ou tipo IIA aparecendo entre as outras fibras (Mascarello *et al.*, 1986). Esta distribuição das fibras nos compartimentos varia de acordo com a espécie e o estágio de crescimento do animal.

Nos mamíferos, de um modo geral, a maioria dos músculos é formada por uma população heterogênea de fibras, constituindo mosaico ou pequenos grupos de fibras de um mesmo tipo. Normalmente, os músculos dos membros anteriores dos mamíferos, possuem maior número de fibras de contração lenta ou tipo I, enquanto nos músculos dos membros posteriores, ocorre o predomínio de fibras de contração rápida ou tipo II (Armstrong & Phelps, 1984). Com isto, tem sido atribuído à função locomotora dos músculos dos membros anteriores, como posturais, e os posteriores como propulsivos (Armstrong *et al.*,

1982; Armstrong & Phelps, 1984). Certos músculos apresentam o predomínio de um certo tipo de fibra sobre outro, como o *extensor digitorum longus* (EDL), músculo branco, predominantemente glicolítico e de contração rápida e, o *soleus* (SOL), músculo vermelho, oxidativo e predominantemente de contração lenta (Crow & Kushmerick, 1982).

1.4. Canais de sódio

Os canais iônicos formam poros através da bicamada lipídica e permitem a íons inorgânicos, principalmente Na^+ , Ca^{2+} , K^+ ou Cl^- , difundirem-se através da membrana, a favor de seus gradientes eletroquímicos. Eles apresentam seletividade iônica, permitindo a passagem de alguns íons inorgânicos, mas não de outros. Esses canais iônicos têm portões ou "gating" que, em geral, abrem-se transitoriamente em resposta a um estímulo específico, tais como uma mudança no potencial de membrana (canais voltagem-dependentes) ou a ligação de um neurotransmissor (canais controlados por um transmissor). As fibras nervosas e as fibras musculares esqueléticas, células eletricamente excitáveis, especializaram-se em usar os canais iônicos voltagem-dependentes para receber, conduzir e transmitir sinais elétricos (Alberts, 1994b).

Os canais de sódio voltagem-dependentes são poros hidrofílicos constituídos por proteínas integrais transmembranas, responsáveis pela modulação da permeabilidade das membranas excitáveis aos íons sódio, e portanto, importantes na geração de potenciais de ação (Hodgkin & Huxley, 1952). Eles podem apresentar-se em três estados funcionais distintos: repouso, ativado ou inativado (Catterall, 1993).

No estado de repouso, a membrana de um axônio, por exemplo, é muito mais permeável aos íons potássio do que aos íons sódio, e deste modo, o potencial de membrana é determinado pela relação entre a concentração de K^+ interna e externa (excesso de cargas positivas no lado externo da membrana e um excesso de cargas negativas no interior). Em células nervosas e musculares esqueléticas, um estímulo suficiente para causar despolarização, provoca imediatamente a abertura de canais de Na^+ -voltagem dependentes, permitindo que, uma pequena quantidade de Na^+ entre na célula a favor de seu gradiente eletroquímico. A entrada de Na^+ despolariza ainda mais a membrana e, conseqüentemente, mais canais de Na^+ são ativados. O "feedback" positivo entre a despolarização e a entrada

de Na^+ leva a uma alteração grande e rápida no potencial de membrana, de cerca de -60 mV para +40 mV em um milissegundo. Os canais de Na^+ tem um mecanismo automático de inativação, que faz os canais fecharem-se rapidamente apesar da membrana ainda estar despolarizada. Eles permanecem inativados até uns poucos milissegundos após o potencial de membrana ter retornado ao seu valor negativo inicial (Alberts, 1994b).

Nem todos os canais de sódio são iguais ou tem o mesmo tamanho. Eles apresentam particularidade. Uma série de estudos nas décadas de 80 e 90 têm-se dedicado à formular modelos estruturais e funcionais para explicar a fisiologia dos canais. Os canais de sódio são estruturalmente constituídos por subunidades denominadas de α , $\beta 1$ e $\beta 2$. Os canais de sódio presentes no órgão elétrico do peixe elétrico *Electrophorus electricus* (o primeiro canal de sódio purificado) é uma cadeia única de 260 kDa (Noda *et al.*, 1984). Os canais de sódio purificados de cérebros de ratos apresentam cadeia α de 260 kDa, $\beta 1$ de 36 kDa e $\beta 2$ de 33 kDa (Catterall, 1993). As seqüências do DNAC dos canais de Na^+ presentes no órgão elétrico do peixe elétrico e no cérebro de rato são 61% semelhantes, indicando a conservação das seqüências de aminoácidos que constituem o canal de sódio durante a evolução (*cf.* Stryer, 1996).

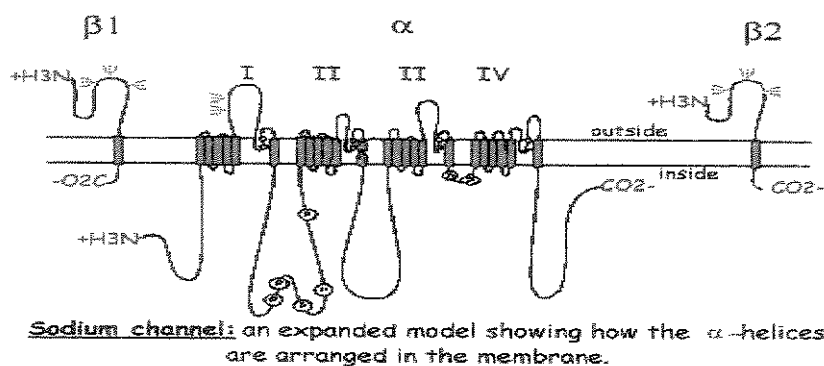


Figura 4: Modelo estrutural do canal de sódio

(<http://www.iacr.bbsrc.ac.uk/notebook/courses/guide/sodiumchannel.html>)

A estrutura primária da subunidade α (Figura 4) é formada por quatro domínios homólogos transmembrana, cada um contendo seis prováveis segmentos em alfa-hélices (S1-S6) com seus terminais amino e carboxil localizados no lado citoplasmático da

membrana. Entre os segmentos S5 e S6, existe uma alça denominada de segmentos SS1 e SS2 que constituem o poro central do canal. O segmento S4 dos quatro domínios homólogos agiria como um “sensor de voltagem”, responsável pelo mecanismo de ativação, isto é, abertura do canal, por possuir aminoácidos de arginina e lisina que apresentam cargas elétricas positivas. Já a região entre os segmentos S6 do domínio III e S1 do domínio IV, formada por aminoácidos com carga elétrica positiva principalmente aminoácido lisina, e localizada no lado citoplasmático da membrana, estaria envolvida no processo de inativação, ou fechamento do canal (Noda, 1993).

O poro iônico do canal de sódio age como um filtro de seletividade, pois apresenta uma dimensão aproximada à do íon sódio, permitindo uma seletividade 12 vezes maior para íons Na^+ do que para outros íons (Catterall, 1993).

Os canais de sódio são fosforilados por proteínas quinases, reguladas de acordo com a concentração de Ca^{2+} intracelular e AMPc e podem ser modulados biossinteticamente (Catterall, 1993).

Mutações, que possam ocorrer nos genes que expressam as proteínas dos canais de sódio, podem afetar a estrutura primária do canal, e em humanos, estas mutações levariam a casos de desordens ou miopatias como: paralisia periódica hipercalêmica, paralisia periódica hipocalêmica, paramiotonia congênita, miotonia flutuante, miotonia permanente, miotonia à resposta de acetazolamida (um diurético inibidor da produção de líquido) e hipertemia maligna (cf. [http:// www.neuro.wustl.edu/neuromuscular/mother/chan.html](http://www.neuro.wustl.edu/neuromuscular/mother/chan.html)).

1.5. Canais de sódio e fibras musculares

Em neurônios e fibras musculares, os canais de sódio voltagem-dependentes que mediam a transmissão sináptica, estão concentrados nas regiões subsinápticas, próximos aos sítios de liberação de neurotransmissores (Hille & Campbell, 1976).

Caldwell e colaboradores (1986), através de estudos eletrofisiológicos, concluíram que há alta concentração de canais de sódio próximo a placa motora de fibras musculares e, esta distribuição de canais diminui à medida que se distancia da placa, sendo baixa próximo à região do tendão.

Vital Brazil & Fontana (1993), analisando os efeitos de toxinas ativadoras de canais de sódio (crotamina, veneno bruto de *Phoneutria nigriventer*, PhTx2 e veratrina) sobre fibras musculares esqueléticas, observaram que estas toxinas produziam maior despolarização na membrana de regiões juncionais (próximo à placa motora) do que nas regiões extrajuncionais, e atribuíram esta diferença na distribuição não uniforme de canais de sódio sobre o sarcolema. Entretanto, a veratridina e a batrachotoxina despolarizaram as regiões juncionais e não-juncionais de maneira semelhante. A explicação encontrada pelos autores seria a possível presença de canais de sódio silenciosos (canais de Na⁺ voltagem-insensíveis) nas regiões extrajuncionais de fibras musculares que seriam ativados pela veratridina e a batrachotoxina.

Há diferença na distribuição de canais de sódio nos diferentes tipos de fibras musculares. As fibras musculares glicolíticas contêm maior concentração de canais de Na⁺ no sarcolema do que as fibras oxidativas (Ruff, 1992; Milton & Behfourouz, 1995). As desigualdades fenotípicas, fisiológicas e bioquímicas existentes em cada tipo de fibra muscular são determinadas pelo tipo de inervação motora presente (Nemeth *et al.*, 1981).

1.6. Toxinas que atuam em canais de sódio

Existem muitas toxinas naturais, de origem animal ou vegetal, que se ligam a sítios específicos presentes nos canais de sódio voltagem-dependentes e alteram a sua fisiologia (ver Tabela). O uso destas toxinas em modelos experimentais vem contribuindo para a melhor compreensão da fisiologia e estrutura dos canais de sódio.

Estas toxinas podem agir na fisiologia dos canais de sódio de três modos: bloqueando-o, retardando-o ou alterando sua inativação ou ainda causando a ativação persistente dos canais. O mecanismo pelo qual o efeito sobre o canal se expressará, dependerá em quais dos sítios de ligação, presentes nos canais de sódio, as toxinas atuarão, se nos sítios responsáveis pela abertura, fechamento, ou ambos.

Tabela: Toxinas que atuam em canais de sódio e seus sítios de ligação

Sítio de ligação	Grupo de Toxinas	Efeitos sobre os canais de sódio
I	Compostos guanidínicos heterocíclicos solúveis em água e conotoxinas	Bloqueiam o influxo de íons sódio
II	Toxinas lipossolúveis	Causam ativação persistente
III	Toxinas α -escorpiônicas e de anêmonas do mar, e atracotoxinas	Retardam a inativação
IV	Toxinas β -escorpiônicas e veneno da aranha <i>Phoneutria nigriventer</i>	Ativam ou retardam a inativação
V	Ciguatoxinas e brevetoxinas	Causam ativação persistente

1.6.1. Toxinas de sítio I

A tetrodotoxina, a saxitoxina e as μ -conotoxinas provocam o bloqueio do fluxo de íons sódio através do canal, de modo reversível, sem alterar o mecanismo de ativação ou inativação voltagem-dependente (Becker & Gordon, 1992). Estas toxinas marinhas são consideradas problemas de saúde pública pois podem provocar quadros de intoxicação alimentar fatal em humanos.

A tetrodotoxina (TTX) é um composto guanidínico heterocíclico, encontrada em ovários, fígado e pele de peixes da subordem *Gymnodontes* conhecidos como peixes “fugu” no Japão, podendo também ser encontrada em algumas espécies de lagartixas, sapos e polvos. Esta toxina atua no sistema respiratório, cardiovascular e neuromuscular (Catterall, 1980). Porém, em músculo cardíaco adulto e músculo esquelético desnervado há presença de canais de sódio TTX insensíveis (Becker & Gordon, 1992).

Os egípcios foram os primeiros a saber que os peixes “fugu” eram venenosos. Em 14 de abril de 1883, Charles Rémy registrou para a Societé de Biologie (Paris) os resultados

do primeiro trabalho experimental com esse peixe. Ele listou cinco espécies de peixes e afirmou que a toxina era encontrada principalmente nas gônadas desses animais. Neste mesmo período, alguns japoneses viajaram para a Europa, principalmente para a Alemanha, com intuito de realizar pesquisas científicas. Retornando ao Japão, D. Takahashi tornou-se o primeiro professor de farmacologia na Universidade de Tóquio e, em 1889, publicou um estudo inédito sobre a farmacologia dessa toxina. Neste estudo, descreveu que a toxina pode causar hipotensão e parada respiratória. Em 1911, Tahara isolou a toxina dos ovários do “fugu” e a denominou de tetrodotoxina. Em 1918, Ishihara mostrou que os nervos sensoriais eram bloqueados pela ação da TTX. Alguns anos mais tarde, Iwakama e Kimura compararam a ação anestésica local da TTX com a da cocaína. Em 1960, Narahashi e colaboradores mostraram que a TTX bloqueava o potencial de ação de fibras musculares devido à inibição do influxo de íons sódio através de estudos de “voltage-clamp” (para revisão ver Fuhrman, 1986).

A saxitoxina (STX) também é um composto guanidínico heterocíclico produzido pelo dinoflagelado pertencente ao gênero *Gonyaulax* e encontrada em grande concentração em moluscos e mexilhões (Catterall, 1980). Em 1965, Kao e Nishiyama (cf. Salgado *et al.*, 1986) propuseram que a TTX e a STX eram moléculas muito grandes para passar através dos canais de sódio, desse modo elas bloqueavam os canais impedindo a passagem de outros íons, agindo nos canais como “uma rolha na garrafa”, com o grupo guanidínio de suas moléculas fazendo o papel de rolha. Em 1975, Hille (cf. Salgado *et al.*, 1986) propôs um modelo para o bloqueio das toxinas, o grupo guanidínio ligar-se-ia ao grupo carboxila do filtro de seletividade do canal de sódio, formando cinco pontes de hidrogênio entre as moléculas das toxinas e o canal. O bloqueio da ligação de TTX no canal de sódio por tetrafluoroborato trimetiloxônio (TMO), um reagente com grupo carboxila modificado, reforçou o modelo proposto por Hille. Entretanto, os canais TTX-resistentes mostravam seletividade normal, sugerindo que o grupo carboxila necessário para que a TTX se ligasse ao canal era distinto daquele presente no filtro de seletividade (para revisão ver Salgado *et al.*, 1986). Noda e col. (1984) criaram um canal de sódio de cérebro de rato mutante através de técnica de mutagênese, e examinaram a sensibilidade do canal à TTX e à STX. Assim, determinaram que estas toxinas se ligam ao sítio 1 (localizado na alça entre os segmentos

transmembrana S5 e S6 do domínio 1 que constituem o poro central do canal de sódio) (para revisão ver Becker & Gordon, 1992).

As conotoxinas (também chamadas de μ -conotoxinas) são polipetídeos isolados do gastrópodo *Conus geographus*. Elas bloqueiam os canais de sódio das fibras musculares de maneira semelhante à da TTX. Competem com a STX pelo mesmo sítio receptor nos canais de sódio (Catterall, 1980; Becker & Gordon, 1992). As $m\mu$ -conotoxinas bloqueiam os canais de sódio presentes nas fibras musculares esqueléticas, sem afetar os canais presentes nos terminais nervosos. A toxina isolada do *Conus consors* (CcTx) e a conotoxina EVIA (denominadas de α -conotoxinas) agem nos canais de sódio de axônios e terminais nervosos, não tendo efeito sobre fibras musculares esqueléticas (para revisão ver Favreau *et al.*, 1999).

1.6.2. Toxinas de sítio II

A veratridina, a aconitina, a batrachotoxina e a grayanotoxina, juntamente com a veratrina, causam a ativação persistente dos canais de sódio, promovendo despolarização e respostas elétricas iterativas em membranas excitáveis de fibras musculares ou nervosas (Macfarlane & Meares, 1958; Ulbricht, 1969). A natureza hidrofóbica destas toxinas sugere que o sítio de ligação delas (sítio 2) encontra-se na região hidrofóbica da membrana, interagindo com as hélices transmembrana do canal de sódio (Becker & Gordon, 1992).

A veratridina é um alcalóide esteróide, o mais potente alcalóide do gênero *Veratrum*, extraído de plantas da subordem Melanthaceae, da família Liliaceae (Benforado, 1967). Esta toxina induz contração muscular, impulsos nervosos repetitivos e arritmias cardíacas. Estes efeitos farmacológicos são provenientes das alterações nos canais de sódio provocado pela toxina, induzindo hiperexcitabilidade, despolarização das membranas excitáveis e aumento da permeabilidade aos íons sódio. A TTX inibe a ação da veratridina de forma não-competitiva, indicando que a veratridina e a TTX agem em sítios diferentes nos canais de sódio (Catterall, 1980).

A batrachotoxina (BTX), substância altamente tóxica, é um alcalóide encontrado na pele de rãs do gênero *Phyllobates*. Os efeitos farmacológicos da BTX resultam da

despolarização de fibras musculares e nervosas causada pelo aumento da permeabilidade aos íons sódio, e estes efeitos são inibidos de forma não-competitiva pela TTX. Esta toxina causa a ativação persistente dos canais de sódio no potencial de repouso da membrana (Catterall, 1980).

A aconitina é um alcalóide encontrado em plantas *Aconitum napellus*. Em preparações utilizando fibras musculares cardíacas, a aconitina causa arritmias e repetitivos pós-potenciais e oscilações seguindo estimulação nervosa. Estes efeitos são provocados pela despolarização prolongada seguida de potenciais de ação, que impede a repolarização completa das membranas excitáveis e esta despolarização pode ser bloqueada pela TTX (Catterall, 1980).

As grayanotoxinas (grayanotoxina I, II e III) são toxinas diterpenóides obtidas de plantas do gênero *Rhododendron*, *Kalmia* e *Leucothoe*, pertencentes à família Ericaceae. As grayanotoxinas II e III são derivadas da I. Estas toxinas possuem mecanismos de ação similares, causando despolarização das membranas excitáveis pela ativação dos canais de sódio em vários graus. A TTX também é um inibidor não-competitivo destas toxinas (Becker & Gordon, 1992).

Experimentos demonstrando interações competitivas com as quatro toxinas lipossolúveis mostraram que a BTX é um agonista total, isto é, ela é capaz de ativar todos os canais de sódio presentes em células de neuroblastoma e que a veratridina, a aconitina e as grayanotoxinas são agonistas parciais (para revisão ver Catterall, 1980).

1.6.3. Toxinas de sítio III

As toxinas α -escorpiônicas e algumas toxinas de anêmonas do mar atuam retardando a inativação dos canais de sódio.

As toxinas isoladas dos venenos de escorpiões norte-africanos (toxinas α -escorpiônicas) são polipeptídeos básicos com peso molecular de aproximadamente 7.000. São proteínas que estão em cadeia única ligada por quatro pontes dissulfeto (Catterall, 1980). Estas toxinas são encontradas nos venenos de escorpiões pertencentes ao gênero *Leiurus*, *Buthus* e *Androctonus* (Lazdunski & Renaud, 1982). As toxinas α -escorpiônicas

causam aumento na secreção de neurotransmissores, arritmias cardíacas, respostas elétricas repetitivas e despolarização em fibras nervosas (Catterall, 1980). A liberação de grandes concentrações de neurotransmissores é a responsável pela maior parte dos sintomas do envenenamento (Vital Brazil, 1986). A toxina mais potente e melhor estudada foi isolada do veneno do escorpião *Leiurus quinquestriatus*. A análise do efeito do veneno do escorpião *L. quinquestriatus*, através de técnica de "voltage-clamp", mostrou que a inativação dos canais de sódio é lenta e incompleta, e a ativação e a inativação são modificadas para potenciais de membrana mais negativos. As toxinas α -escorpiônicas não causam ativação persistente dos canais de sódio, mas podem aumentar a ativação provocada pelas toxinas lipossolúveis. Elas aumentam a fração de canais de sódio ativados pelos agonistas parciais (veratridina, aconitina e grayanotoxinas). Esta interação cooperativa entre as toxinas lipossolúveis e as toxinas α -escorpiônicas indica que elas agem em receptores separados mas que interagem alostericamente (Catterall, 1980). Em 1986, Noda e col. propuseram que a toxina V de *L. quinquestriatus* (LqV) se ligaria à alça extracelular entre os segmentos S5 e S6 da subunidade α do canal de sódio (sítio 3) e, interagiria com o segmento S4 (sensor de voltagem do canal), retardando ou prevenindo a mudança conformacional do estado ativo para o inativo do canal (cf. Becker & Gordon, 1992).

As toxinas de anêmonas do mar são polipeptídeos básicos com 27 a 51 aminoácidos, e são isoladas de anêmonas pertencentes aos gêneros *Anemonia*, *Anthopleura* e *Radianthus* (Catterall, 1980; Lazdunski & Renaud, 1982). As toxinas I e II isoladas da *Anemonia sulcata* e a anthopleurina A da *Anthopleura xanthogrammica* possuem 47-49 aminoácidos e tem seqüências homólogas. A toxina III da *A. sulcata* tem apenas 27 aminoácidos e também algumas seqüências homólogas com as duas outras toxinas. Entretanto, as toxinas de anêmonas não possuem seqüências homólogas com as estruturas das toxinas α -escorpiônicas. Os mecanismos de ação destas toxinas são similares aos das toxinas α -escorpiônicas: retardam a inativação dos canais de sódio, aumentam a ativação persistente do canal provocada pelas toxinas lipossolúveis e se ligam ao mesmo sítio (Catterall, 1980).

As aranhas pertencentes à subfamília Atracinae, compreendem cerca de 35 espécies divididas entre os gêneros *Atrax* e *Hadronyche*, e são encontradas principalmente no litoral sudeste australiano. Alguns polipeptídeos conhecidos como atracotoxinas (ACTX) foram isolados do veneno dessas aranhas. A δ -ACTX-Hv1, conhecida por versutoxina e isolada do veneno da aranha *Hadronyche versuta*, e a δ -ACTX-Ar1, conhecida por robustoxina e isolada do veneno da *Atrax robustus*, retardam a inativação dos canais de sódio sensíveis à TTX, de maneira semelhante à ação das toxinas α -escorpiônicas e das toxinas isoladas de anêmonas-do-mar. Estas toxinas têm cerca de 42 aminoácidos e possuem seqüências homólogas. As δ -ACTXs representam uma nova classe de toxinas que interagem com canais de sódio presentes em mamíferos e em insetos e se ligam a sítios similares ao sítio 3, local de ligação das toxinas α -escorpiônicas e das toxinas de anêmonas-do-mar. Nem todas as toxinas são prejudiciais ao homem, algumas podendo trazer benefícios. Por exemplo, a δ -ACTX-Hv1b apresenta alta toxicidade para insetos, e pouca ação para mamíferos, sendo assim, esta fração tóxica pode ser utilizada como um biopesticida (cf. Szeto *et al.*, 2000).

1.6.4. Toxinas de sítio IV

As toxinas β -escorpiônicas são polipeptídeos básicos com massa molecular de aproximadamente 7000 daltons, isoladas de escorpiões americanos pertencentes aos gêneros *Tityus* e *Centruroides*. Apresentam seqüências homólogas com as toxinas α -escorpiônicas. Estas toxinas causam alteração na voltagem da ativação do canal de sódio de 40-50 mV para potenciais de membrana mais negativos, conseqüentemente modificando a inativação. Ligam-se ao sítio 4 (localizado no lado extracelular) dos canais de sódio (para revisão ver Catterall, 1980; Honerjäger, 1982 ; Becker & Gordon, 1992).

A fração tóxica 2, isolada do veneno bruto da aranha armadeira *Phoneutria nigriventer* (PhTx2), atua na ativação dos canais de sódio presentes em fibras musculares esqueléticas e nervosas (Vital Brazil & Fontana, 1993) e, provavelmente no mesmo sítio em que as toxinas β -escorpiônicas se ligam aos canais (Vital Brazil, 1986). A ação da PhTx 2 é inibida pela TTX.

1.6.5. Toxinas de sítio V

Existem as ciguatoxinas e brevetoxinas que são responsáveis por causar intoxicações em humanos, caracterizadas principalmente por distúrbios neurológicos. Estas toxinas são produzidas pelos dinoflagelados pertencentes às espécies *Gambierdiscus toxicus* e *Ptychodiscus brevis* e afetam fibras nervosas mielínicas e junções neuromusculares de vertebrados. Ligam-se ao sítio 5, localizado na subunidade α dos canais de sódio. Estudos eletrofisiológicos identificaram essas toxinas como ativadoras dos canais de sódio. Elas aumentam o fluxo de íons sódio em células excitáveis, conseqüentemente provocando descargas repetitivas de potenciais de ação (Mattei *et al.*, 1999).

1.6.6. Outras toxinas que atuam em canais de sódio

A crotamina, uma toxina isolada da peçonha da cascavel sul-americana, *Crotalus durissus terrificus*, é um polipeptídeo básico composto por 42 aminoácidos. Ela provoca despolarização nas membranas de fibras musculares e aumenta a permeabilidade aos íons sódio (Chang & Tseng, 1978). Pode também inibir a transição dos canais de sódio do estado de repouso para inativado, permitindo que os canais em repouso sejam automaticamente ativados (Matavel *et al.*, 1998).

A miotoxina α é um polipeptídeo básico com cerca de 39 aminoácidos, isolado da peçonha da cascavel norte-americana *Crotalus viridis viridis* (Cameron & Tu, 1978). Esta toxina causa despolarização do sarcolema e induz fasciculação do músculo com potenciais de ação repetitivos. Há controvérsias sobre o sítio de ação desta toxina. Hong & Chang (1985) sugeriram que a miotoxina α age em sítios localizados no sarcolema e túbulos-T, que diferem dos sítios de ligação de outras neurotoxinas. Estes sítios estariam possivelmente localizados nos canais de Na^+ voltagem-dependentes e a mitoxina α atuaria no mecanismo de ativação dos canais. A ativação dos canais de sódio induzida pela toxina aumentaria o influxo de Na^+ e fluido extracelular, possibilitando também o aumento na concentração intracelular de cálcio via trocador $\text{Na}^+-\text{Ca}^{2+}$. Esses autores propuseram também que a miotoxina α e a crotamina agiriam num mesmo sítio de ligação devido às semelhanças químicas e farmacológicas (além de imunológicas) entre ambas. Entretanto,

Volpe e colaboradores (1986) demonstraram, através de ensaios imunoquímicos e bioquímicos utilizando vesículas de retículo sarcoplasmático isoladas, que a miotoxina α provoca o desacoplamento da bomba Ca^{2+} -ATPase, proteína localizada na membrana do retículo sarcoplasmático, inibindo a recaptção de Ca^{2+} . Conseqüentemente, a miotoxina α causaria um aumento na concentração intracelular de Ca^{2+} , justificando as alterações morfológicas, tais como miovacuolização observadas por outros autores (Ownby *et al.*, 1976; Cameron & Tu, 1978; Hong & Chang, 1985). A TTX é um antagonista não-competitivo das toxinas crotamina e miotoxina α .

1.7. Toxinas ativadoras de canais de sódio e danos neuromusculares

Existem poucos estudos histológicos e ultraestruturais, envolvendo toxinas que agem em canais de sódio.

Em estudo experimental *in vitro* com a batrachotoxina, foi observado que esta toxina produzia alterações estruturais em fibras musculares e nervosas (Albuquerque *et al.*, 1971). Esta toxina induzia dilatação das cisternas terminais do retículo sarcoplasmático. Podia também ocasionar a ruptura dos túbulos-T e do retículo sarcoplasmático, dependendo da dose (Warnick *et al.*, 1971). A batrachotoxina, injetada dentro do nervo ciático, provocava também edema na região dos nodos de Ranvier e região paranodal de fibras nervosas mielínicas associado com redução no calibre axonal nas regiões internodais. Os efeitos eram causados pelo fluido intracelular excessivo induzido pelo aumento da permeabilidade aos íons sódio e aumento na densidade de organelas axoplasmáticas (Wayne Moore *et al.*, 1986).

Alterações estruturais nas fibras nervosas, similares às observadas no estudo com a batrachotoxina, já tinham sido encontradas no experimento *in vivo* com o veneno bruto da aranha *Phoneutria nigriventer* em nervo ciático de camundongos (Cruz-Höfling *et al.*, 1985). Os autores observaram a ocorrência de edema no axoplasma nodal e paranodal, desenvolvimento de vacúolos no espaço periaxonal, aumento na densidade de organelas axoplasmáticas e redução no calibre axonal das regiões internodais. Eles atribuíram como causa destas anormalidades nas fibras nervosas mielínicas, o influxo de íons sódio no nodo de Ranvier provocado pela ação do veneno sobre os canais de sódio. Os venenos dos

escorpiões *Leiurus quinquestriatus* e *Centruroides sculpturatus* causaram alterações morfológicas idênticas àquelas produzidas pelo veneno da *Phoneutria*. Essas alterações não se manifestaram com o pré-tratamento com TTX (Love *et al.*, 1986).

As ciguatoxinas e as brevetoxinas fazem parte também da classe das neurotoxinas que ativam canais de sódio. Causam edema em nodos de Ranvier de fibras nervosas mielínicas e em placas motoras de fibras musculares (Mattei *et al.*, 1999).

Ownby e colaboradores (1976) demonstraram através de análise ao microscópio eletrônico que a miotoxina α , toxina isolada da peçonha de *Crotalus viridis viridis*, induz dilatação do retículo sarcoplasmático, desorganização com dissociação de miofibrilas e dano mitocondrial quando injetada via intramuscular em camundongos. Estas alterações poderiam ser causadas pelo acúmulo intracelular de íons Na^+ resultantes da ativação dos canais de Na^+ induzida pela toxina, ou pelo aumento intracelular de íons Ca^{2+} devido à ação da toxina no trocador $\text{Na}^+-\text{Ca}^{2+}$ (Hong & Chang, 1985) ou na bomba Ca^{2+} -ATPase localizada no retículo sarcoplasmático (Volpe *et al.*, 1986).

Em estudo experimental utilizando a miotoxina α e a crotamina, fração tóxica do veneno de *Crotalus durissus terrificus*, foi observado que a miovacuolização causada por estas toxinas eram qualitativamente similares, entretanto o local da miovacuolização não pode ser determinado através de microscopia de luz (Cameron & Tu, 1978). Chang & Tseng (1978) demonstraram, através de estudos miográficos e eletrofisiológicos, que a crotamina provocava uma despolarização nas membranas de fibras musculares e, que esta despolarização era abolida com um pré-tratamento com TTX ou com baixa concentração de íons Na^+ no meio de incubação. O antagonismo provocado pela adição de íons Ca^{2+} ao meio sugeria o envolvimento de correntes de cálcio no mecanismo de ação da crotamina, além do aumento de influxo de Na^+ .

Recentemente, Mattiello-Sverzut & Cruz-Höfling (2000), através de microscopia de luz e eletrônica de transmissão, mostraram que a PhTx2 causa alterações morfológicas em nervo frênico e músculo diafragma de camundongos, *in vitro*, como, hipercontração de miofibrilas, desorganização de sarcômeros, dilatações de cisternas terminais do retículo sarcoplasmático, depleção de vesículas nas placas motoras, vacuolização de axônios e células de Schwann, dano mitocondrial e mionecrose. Estas alterações foram atribuídas aos

distúrbios osmóticos ocasionados pelo aumento do influxo de íons sódio induzido pela toxina.

A veratrina pode ocasionar também miovacuolização. Em experimento *in vitro*, utilizando o músculo diafragma de camundongos, Cruz-Höfling & Vital Brazil (1987, 1990) mostraram através de análise ultraestrutural que os vacúolos encontrados em algumas fibras musculares eram dilatações das cisternas do retículo sarcoplasmático. Os autores atribuíram como causa dessas dilatações, o aumento do influxo de íons sódio para o interior das células provocado pela veratrina, uma vez que não se manifestavam quando a preparação era previamente incubada com TTX.

1.8. Objetivos

Considerando:

- que nosso modelo experimental enfocou os efeitos da veratrina, alcalóide que ativa os canais de sódio sobre células musculares esqueléticas;
- que pretendíamos investigar se haviam diferenças entre a resposta dada pelos diferentes tipos de fibras musculares – oxidativas, oxidativa-glicolíticas e glicolíticas – em dois tipos de músculos de mamíferos, o sóleo (de contração lenta e predominantemente com metabolismo oxidativo), e o extensor longo dos dedos (de contração rápida, predominantemente glicolítico);
- que pretendíamos verificar se a resposta dada pelo mesmo tipo de fibra era igual ou diferente, dependendo de a mesma estar inserida no músculo sóleo ou no músculo extensor longo dos dedos;
- que pretendíamos verificar se a interação da veratrina com esses tipos de fibras musculares esqueléticas de mamíferos (camundongos) era equivalente à exibida pelos mesmos tipos de fibras em músculo esquelético de peixes (tilápias);

- que pretendíamos observar os efeitos da veratrina sobre a ultraestrutura das fibras musculares do sóleo e extensor longo dos dedos de camundongos BALB/c adultos, e do músculo lateral da tilápia, *Oreochromis niloticus*, com o objetivo de identificarmos quais as organelas-alvo principalmente atingidas pela veratrina, e se havia diferença entre os três tipos de músculos;
- que a veratrina é um alcalóide de natureza lipídica, extraído de plantas (*Schoenocaulon officinale*), pertencentes ao grupo dos compostos policíclicos lipossolúveis, que, uma vez em contato com membranas excitáveis como o sarcolema, promove a ativação de canais de sódio, fazendo com que permaneçam abertos por mais tempo, e, conseqüentemente, permitindo que o influxo de Na^+ seja aumentado durante a despolarização da membrana;
- que a veratrina promove respostas iterativas (despolarizações repetitivas) da fibra muscular, e que esses efeitos devem ter conseqüências que podem estar refletidas na atividade oxidativa, na atividade contrátil e nas características ultraestruturais das fibras musculares;
- que o aumento citossólico de Na^+ leva a alterações no funcionamento de canais de Ca^{2+} ;
- que dentro desse contexto era relevante investigar-se possíveis efeitos da veratrina sobre mitocôndrias isoladas de músculo esquelético, já que essas organelas não só tem papel definitivo no metabolismo da fibra muscular “condicionando-a” à atividade predominantemente aeróbica, ou anaeróbica (dependendo de seu maior ou menor número), mas também possui papel tampão no metabolismo do cálcio celular (junto com o retículo sarcoplasmático);
- que a utilização de outras toxinas com ação antagônica a da veratrina, como por exemplo a tetrodotoxina (TTX), que bloqueia canais de Na^+ , possibilitam a comprovação dos efeitos promovidos pela primeira, quando utilizadas como pré-tratamento;

Estabelecemos como modelo experimental, o estudo in vivo da ação da veratrina sobre os músculos sóleo e EDL de camundongos BALB/c, e o músculo lateral da tilápia Oreochromis niloticus, através de estudos histoenzimológicos por meio das reações NADH-TR e m-ATPase, e através da microscopia eletrônica de transmissão para caracterização das alterações ultraestruturais induzidas pelo alcalóide, bem como o estudo in vitro dos efeitos da veratrina sobre mitocôndrias isoladas.

RESULTADOS: ORGANIZAÇÃO

O projeto de pesquisa desenvolvido para a obtenção do título de Mestre deu origem a quatro trabalhos científicos, três dos quais, prontos para serem submetidos à publicação. O quarto trabalho ainda requer que sejam feitos alguns experimentos adicionais, para estar apto a ser submetido.

Considerando os aspectos relacionados à abordagem metodológica empregada na investigação, os enfoques utilizados para elucidar pontos relativos à interação da veratrina com o tecido muscular, os tipos de animais da experimentação, e, ainda, considerando os aspectos relativos às dimensões, em termos de páginas, que seria razoável o trabalho possuir, foi feita a apresentação dos resultados da seguinte forma:

- No artigo 1 foram enfocados os resultados da interação do alcalóide veratrina com os músculos sóleo e extensor longo dos dedos, após administração in situ, particularmente no que se refere à expressão fenotípica exibida pelos tipos de fibras musculares esqueléticas, revelada por métodos histoenzimológicos para detecção da atividade oxidativa (método NADH-TR) e da atividade contrátil (reação ATPase-miosínica em pH ácido e alcalino). Os resultados revelaram que a veratrina é capaz de modular a atividade metabólica das fibras, alterar a proporção dos tipos de fibras e induzir no sóleo o aparecimento de um terceiro tipo. O comportamento de um mesmo tipo de fibra é diferente frente à veratrina, dependendo da sua localização ser no sóleo ou no EDL.*
- No artigo 2 foram enfocadas as alterações ultraestruturais induzidas pela veratrina após administração in situ no músculo sóleo ou no extensor longo dos dedos, destacando aqueles aspectos que se configuravam como distintos da interação veratrina-sóleo e veratrina-EDL. Os resultados foram discutidos à luz das características farmacológicas-fisiológicas da veratrina e bioquímicas-fisiológicas de cada músculo e levantando questões sobre a entrada ou não do alcalóide dentro da célula e algumas consequências advindas dessas possibilidades. O pré-tratamento com tetrodotoxina, que bloqueia os canais de sódio nos permitiu comprovar que as alterações ultraestruturais decorriam da ação da veratrina nos canais.*

- *No artigo 3 foram abordados os efeitos da veratrina sobre o músculo lateral da tilápia, tanto sob o ponto de vista das alterações ultraestruturais, como das atividades do metabolismo oxidativo e contrátil dos diferentes tipos de fibras musculares evidenciadas pelas reações NADH-TR e m-ATPase. A discussão dos resultados abordou os dados histoenzimológicos destacando os dados inesperados com relação a diferenças regionais exibidas pela atividade oxidativa dentro da mesma fibra, bem como aparecimento de um quarto tipo de fibra não observado nas tilápias controle. Os resultados histoenzimológicos, como os ultraestruturais observados no sóleo e EDL de camundongos, permitiram verificar que, muito embora a veratrina deva atuar igualmente na fisiologia dos canais de sódio do sarcolema de músculo esquelético de peixes, algumas especificidades do mecanismo de ação devem existir, uma vez que diferenças substantivas foram detectadas entre ambos os modelos experimentais.*
- *No artigo 4 redirecionamos nosso foco de investigação, particularizando nossa atenção sobre as mitocôndrias isoladas de músculos esqueléticos quanto aos efeitos da veratrina. Foram utilizadas mitocôndrias isoladas dos músculos das pernas traseiras de camundongos ou de ratos e mitocôndrias de fígado de ratos. Os resultados até agora obtidos apontam para o efeito inibitório da veratrina sobre a respiração mitocondrial em mitocôndrias hepáticas e efeito no aumento da permeabilidade a prótons da membrana interna das mitocôndrias de músculos. Reputamos a uma possível internalização da veratrina nas fibras musculares como potencial responsável pelas alterações descritas nos Artigos 1 e 2. Este efeito somado as alterações nas propriedades de permeabilidade iônica seriam os responsáveis pelos efeitos observados.*

Nossos resultados lançam luzes sobre aspectos que ainda não tinham sido abordados da interação veratrina-músculo esquelético e reforçam a idéia da utilização de toxinas com ação altamente específica, que se prestam como ferramentas valiosas no esclarecimento de processos biológicos como os que aqui foram investigados.

✉ Maria Alice da Cruz-Höfling
Departamento de Histologia e Embriologia
Instituto de Biologia, C.P. 6109
Universidade Estadual de Campinas (UNICAMP),
13 087-130, Campinas – SP – BRASIL
email: hofling@obelix.unicamp.br
Tel. (55)(19)3788 7801; Fax. (55)(19)3289 1951

**A HISTOCHEMICAL STUDY OF THE INTERACTION OF MOUSE *EXTENSOR*
DIGITORUM LONGUS AND *SOLEUS* MUSCLES WITH VERATRINE**

¹Erika Maria Silva Freitas, ²Maeli Dal Pai Silva, ✉¹Maria Alice da Cruz Höfling

¹Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, ²Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP), Botucatu, SP, Brasil

Running Title: Muscle histochemical differences induced by veratrine

ABSTRACT

The aim of this study was to compare the morphological changes in the *extensor digitorum longus* (EDL) and *soleus* (SOL) muscles of mice after the intramuscular injection of veratrine (10 ng/kg), a Na⁺-channel activating alkaloid from the plant *Shoenocaulon officinale* (Liliaceae). Fifteen, 30 and 60 min after the injection of veratrine, the mice were sacrificed and the muscles processed for evaluation by standard histochemical methods to determine the oxidative and myosin-ATPase activities. The damage to slow-twitch oxidative, fast-twitch oxidative glycolytic and fast-twitch glycolytic fibers, as well as the changes in fiber type, were assessed. Veratrine altered the metabolic and contractile activities of EDL and SOL. Oxidative fibers and oxidative-glycolytic fibers were more sensitive to veratrine than glycolytic fibers, with both showing a decrease in their metabolic activity. In EDL, the m-ATPase reaction revealed a significant decrease (50%) in the number of type IIB fibers after 30 min while the number of type I fibers increased by 55%. The proportion of type I fibers decreased from 34% in control SOL to 17% in veratrinized muscles, with a 10% decrease in type IIA fibers within 15 min. Concomitantly, a third type of fiber appeared which accounted for 28% of the fibers. The changes in fiber type were maximal by 15 min after veratrine administration. The alterations in EDL may be related to the higher number of Na⁺ channels present in this muscle whereas those in SOL may involve an action of veratrine on mitochondria.

Key words: EDL, fiber type, mouse, Na⁺ channels, soleus muscle, veratrine

INTRODUCTION

Naturally occurring neurotoxic and myotoxic substances are valuable tools for studying pathophysiological phenomena in neuromuscular disorders. Numerous natural toxins from plants and animal venoms act by altering the kinetics of sodium channel activation and inactivation in excitable membranes (4). Despite sharing a common target (Na^+ channels), these substances comprise a heterogeneous group which includes water soluble heterocyclic guanidines, lipid-soluble polycyclic compounds, and small, highly basic peptides, each with a characteristic physiological action.

Na^+ channel-blocking toxins produce no morphological changes as part of their physiological action. In contrast, extensive morphological alterations occur with toxins which activate or delay the inactivation of Na^+ channels, as shown for small basic peptides such as crotamine and myotoxin *a* from *Crotalus durissus terrificus* and *C. viridis viridis* snake venoms, respectively (3, 22).

The morphophysiological effects of lipid-soluble polycyclic compounds such as batrachotoxin (29) and veratrine (5, 6), a commercial extract of the plant *Schoenocaulon officinale*, have been described. These substances cause necrosis in skeletal muscle cells, the onset of which is characterized by multifocal-vacuolation as a result of an osmolarity imbalance caused by an increased influx of Na^+ followed by water. The mechanism behind these alterations is still poorly understood (15, 16). Not all cells in a given muscle undergo myonecrosis following exposure to these substances. Although varying degrees of damage may reflect an uneven diffusion of the toxic agent or an inaccessibility of some fibers to the substance, the nature of the predominant cell type, i.e., glycolytic and/or oxidative, may also be an important factor.

In this work, we used histochemical analysis to examine whether veratrine, a Na^+ channel-activating alkaloid (26), exerts different myotoxic effects in glycolytic and oxidative muscle fibers. The two muscles examined were the *soleus* (SOL), composed mainly of oxidative fibers, and the *extensor digitorum longus* (EDL), which consists mainly of fast-twitch glycolytic fibers.

MATERIALS AND METHODS

Experimental groups

Twelve adult male BALB/c mice (20-25 g) obtained from the Central Animal House at the State University of Campinas were used. The mice were housed in standard plastic cages at 25 °C and had access to food and water *ad libitum*. For the experiments, the mice were divided into four groups (3 mice/group), one of which received sterile saline and the other three, veratrine. For use, the mice were anesthetized with sodium pentobarbital (40-70 mg/kg, i.p.) and the SOL and EDL then exposed prior to the injection of veratrine (5 µl, 10ng/kg - Sigma) into each muscle. The anesthetized mice were sacrificed by cervical dislocation 15, 30 or 60 min later and the muscles excised and their distal portions removed. The remaining central portion of each muscle was then quickly frozen in liquid N₂ and stored at - 80°C. The control group received sterile saline solution under the same experimental protocol.

Histochemistry

Frozen sections (5 µm thick) were obtained with a cryostat and immediately stained with H.E. and Gomori trichrome (10) for analysis of the general muscle fiber morphology. Nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) (9, 23) and myosin-ATPase reactions after acidic or alkaline preincubations (m-ATPase, pH 4.35, 4.6 or pH 10.3) (2, 12) were used to classify the fibers according to their oxidative (metabolic) and contractile activities, respectively. Quantification of the fiber types was provided in saline- and veratrine-treated muscles at 15, 30 or 60 min, in order to examine if the alkaloid affects differently each fiber type.

Statistical methods

Statistical analyses were done using SAS system statistical software*. The procedures used included general linear models (GLM) and Duncan's multiple range test for variables, and the chi-square test for comparing the proportion of fiber types, identified by the m-ATPase reaction, *versus* time.

*SAS System Inc. SAS/STAT[®] User's Guide, Release 6.03 Edition, Cary, NC: SAS Institute Inc., 1998.

RESULTS

Controls

H.E.: - Light microscopy of EDL and SOL from saline-treated (control) mice showed polygonal, regular-sized muscle fibers with a normal morphology (Figs. 1 and 2). In EDL, some fibers were highly oxidative while others showed moderate to pale staining for oxidative activity using the NADH-TR reaction. Using the m-ATPase reaction at pH 4.6, three fiber types were identified in control EDL: dark staining fibers (type I), rarely present in normal muscle, medium to pale staining fibers (type IIA) and pale staining fibers (type IIB). At pH 10.3, type I fibers stained pale and type IIA and IIB fibers showed moderate to intense staining with the m-ATPase reaction. In control SOL, the muscle fibers showed moderate to high oxidative enzyme activity using the NADH-TR reaction. With the m-ATPase reaction (pH 4.6), only dark staining fibers (I) and medium staining fibers (IIA) were identified in control SOL. However, these muscle fibers showed a reversed staining pattern at pH 10.3. Quantification of the fiber types showed that 73% of the EDL fibers were IIB, 22% were IIA and 5% were I; in the SOL, 66% of the fibers were type IIA and 34% were type I (Fig. 3A - B).

Veratrine-treated EDL

H.E. and Gomori: - Within 15 min of veratrine injection, there were signs of muscle tissue injury and differences in the metabolic activity of the EDL and SOL. Qualitatively, the pattern of morphological damage was similar up to 60 min, although the proportion of injured cells increased. In veratrine-treated EDL, the affected fibers showed swelling, delta lesions, and were interspersed with other apparently intact fibers (Fig. 4A). In addition, from 30 min onwards, there was interstitial edema (Fig. 4B). By 60 min, the cells had increased markedly in diameter (Fig. 4C), probably as a result of a greater influx of Na^+ followed by water, and some fibers showed zones of hypercontraction.

NADH-TR: - Comparison of the NADH-TR reaction in saline- and veratrine-treated mice showed that the oxidative fibers in saline-injected EDL had a formazan precipitate that appeared as dark bands in the subsarcolemmal region and as punctuations (or short lines, in oblique cell sections) scattered within the fibers. This reactivity represented

clusters of closely-packed mitochondria immediately under the sarcolemma or a ring of mitochondria arranged around the myofibrils, respectively (Fig. 1B). The glycolytic fibers of control EDL typically showed weak reactivity in the NADH-TR reaction and no reactivity in the subsarcolemmal cytoplasm (Fig. 1B). After the i.m. injection of veratrine, the pattern of the reaction changed and there was a time-dependent reduction reactivity (Fig. 4D-F). The subsarcolemmal black band of formazan particles was thinner and gradually showed discontinuities up to 60 min. Concomitantly, the NADH-TR reaction decreased progressively during the 60 min of observation, with some of the cells showing no reactivity in the mitochondria encircling the myofibrils. Veratrine also abolished the rare, tiny formazan particles seen in control glycolytic fibers (Fig. 4D-F).

m-ATPase: - In the EDL, most of the type I, IIA and IIB muscle fibers showed a normal staining profile with the m-ATPase reaction after acidic and alkaline preincubations (Fig. 5). However, some fibers showed no differentiation as there was no reversal of staining at pH 10.3 (Fig. 5A-F). A number of type I and type II fibers stained alike at pH 10.3 after treatment with veratrine for 30 min and 60 min. In addition, the intensity of the reaction differed from that in control cells after acidic and alkaline preincubations. Quantification of the fiber types showed that in the EDL veratrine significantly decreased (50%) the number of type IIB fibers, and significantly increased (55%) the number of type I fibers ($P = 0.001$), both of which were maximal after 15 min (Fig. 3A).

Veratrine-treated SOL

H.E. and Gomori: - As with EDL, some dark, swollen fibers and a diffuse interstitial edema were seen in the SOL at all periods. By 30 min after veratrine, the signs of fiber injury included a number of split fibers, centralized nuclei, zones of hypercontracted myofibrils, an irregular cell outline and some disintegrating cells (Fig. 6A - C). SOL muscle cells generally showed more alterations typical of artefacts produced by ice crystals (Fig. 4A-C and 6A-C) than the EDL. These changes were attributed to the ability of veratrine to increase the influx of fluid secondary to an increase in Na^+ influx. None of the muscles from control mice group showed such alterations (Figs. 1A and 2A).

NADH-TR: - As with EDL, oxidative and oxidative-glycolytic fibers of SOL showed an irregular distribution of formazan precipitates after exposure to veratrine. Similarly, there was a decrease in the intensity of the reaction together with marked swelling (*cf.* Fig. 2B and Fig. 6D-F for the effect of veratrine on fiber diameter). A considerable number of oxidative fibers showed weak reactivity or had aberrant deposits of formazan. Numerous fibers also appeared partially destroyed (Fig. 6D-F).

m-ATPase:- The m-ATPase reaction (pH 4.6 or pH 10.3) stained the fibers abnormally and three types of fibers, rather than the usual two, were seen in veratrinized SOL muscle (Fig. 7A-F). This "third" type fiber showed no reversal of staining in the acidic or alkaline preincubations. The intensity of the m-ATPase reaction varied with the length of exposure to veratrine and differed from that of control mice. Post-veratrine counting of the SOL fiber types showed that there was a significant decrease ($P = 0.007$) in type I fibers within 15 min, but which then remained constant for up to 60 min after application of the toxin. The "third" fiber type accounted for 28%, 19% and 21% of the total number of fibers after 15, 30 and 60 min, respectively (Figs. 3B). This fiber type apparently mainly from a change in the contractile activity of slow-twitch oxidative fibers (type I), with type IIA fibers contributing only 10% to this new fiber type.

UNICAMP
BIBLIOTECA CENTRAL
SEÇÃO CIRCULANTE

DISCUSSION

Veratrine produced myotoxic effects in the EDL and SOL of mice. Acute signs of injury, such as vacuolated fibers, hypercontraction, swollen cells, delta lesions and interstitial edema were seen as early as 15 min after alkaloid administration. The vacuolation of muscle cells is a common finding with Na^+ channel-activating myotoxins (3, 15, 16, 22) and probably results from changes in sarcolemmal permeability. The EDL and SOL, vacuolation produced by veratrine may involve sarcoplasmic reticulum terminal cisternae swelling without affecting T-tubules (6). The prolonged influx of Na^+ would in turn lead to the accumulation of fluid within the cell reticulum by osmotic forces. An indirect evidence of such effect is offered by technical artifacts-like structures, actually fluid containing-SR vacuoles submitted to rapid freezing when the muscle piece was immersed in liquid N_2 .

The other myonecrotic changes observed in the muscles normally occur following rupture of the sarcolemma. Since, like other Na^+ channel-activating myotoxins, veratrine has no enzymatic or cytolytic activity, the rupture of the cell membrane was considered to be caused by the mechanical forces produced by hydrostatic pressure. As early as 30 min after the injection of veratrine, SOL muscle unexpectedly showed long-term (chronic) signs of damage, such as split fibers (19, 30) and fibers with central nuclei. Saline-treated *soleus* were negative for such types of damage. The mechanism of these phenomena is unknown, but probably involved an imbalance in osmolarity.

Veratrine altered the metabolic and contractile activities of EDL and SOL muscle fibers, as revealed by histochemical procedures. The oxidative and oxidative-glycolytic fibers showed a greater decrease in oxidative enzyme reactions after veratrine than did the glycolytic fibers in both muscles. Veratrine uncouples the respiratory chain in isolated mitochondria from rat and mouse skeletal muscle (unpublished observations). In support of a direct effect of veratrine on oxidative enzyme reactions, veratridine (and supposedly veratrine) (1, 27) binds to site 2 of the Na^+ channel and reaches the cell cytoplasm by diffusing across the membrane in its uncharged form (13). Such internalization of the alkaloid would allow a direct effect on mitochondria and could explain the decrease in the

NADH-TR reaction at all time points after treatment with veratrine. Obviously, hydroelectrolytic disturbances caused by the alkaloid need to be considered when explaining metabolic-induced changes. In this regard, SOL muscle was more affected than EDL.

The appearance of a “third” fiber type detected by the m-ATPase reaction after acidic and alkaline preincubations (SOL), as well as the change in the intensity of the reaction in EDL and SOL muscles after veratrine, suggested that other factors in addition to a hydroelectrolytic disturbance, affected this contractile activity. The activation of Na^+ channels by veratrine leads to repetitive firing in nerve and muscle (1), probably because of prolonged unequal depolarization of the cell membrane (28). The resulting Na^+ overload in the cells will impair the regulation of ion levels critical for normal muscle functioning. An elevation in intracellular $[\text{Ca}^{2+}]$ in muscle fibers as a result of such excitation could contribute to muscle injury. Calcium entry can occur following rupture of the sarcolemma and/or through T-tubule depolarization and Ca^{2+} release from the sarcoplasmic reticulum, all of which may be induced by veratrine in muscle cells.

Florendo et al. (11) investigating the eletrophysiologic differences between EDL and SOL of mice (Bar Harbor 129 strain) verified that EDL contains 60% fast-twitch glycolytic fibers (FG), 39% fast-twitch oxidative-glycolytic fibers (FOG), and 1% slow-twitch oxidative fibers (SO), whereas the SOL contains 50% type FOG fibers and 50% SO fibers. In addition, the authors found that EDL has a higher frequency of miniature end-plate potentials than SOL, which indicates a greater number of acetylcholine molecules combining with acetylcholine receptors per unit time. The action potentials in EDL have a greater amplitude and shorter duration than in SOL, probably because of the greater Na^+ concentration gradient in EDL, which provides a larger electromotive force to generate an action potential of greater amplitude. The difference in Na^+ content between the EDL and SOL may be related to the different fiber types in these muscles (11). Fast fibers have a higher density of Na^+ channels at the neuromuscular junction than slow fibers (18, 24). As a result, EDL shows a higher influx of Na^+ at a lower frequency of external stimulation or during contractile activity than does SOL (20).

Although EDL was more sensitive regarding the proportion of fiber types than SOL to the action of veratrine, the latter muscle showed more severe morphological changes. However, quantification of the fiber types in the SOL indicated that type I fibers contributed more to the appearance of a third fiber type (indicated by asterisks in Fig. 7), since there was a significant decrease in slow-twitch oxidative fibers. The 50% decrease in type I fibers and the appearance of split fibers or fibers with central nuclei suggested that the third fiber type seen with the m-ATPase reaction in veratrinized animals may derive from a change in the contractile activity of type I fibers in the SOL. These cells would form undifferentiated-like or type IIC-like fibers (with no reversal of staining in the two pHs) which are common in exercise-induced muscle damage (30), in chronic electrical stimulation (14) and after snake venom (7) or myotoxin inoculation (19, 25). The damage caused by these stimuli and agents as those above mentioned, requires a larger time scale than the rapid effects (within 60 min) seen in this study, a clear understanding for these findings remains to be clarified.

Oxidative fibers, which have a high concentration of mitochondria and are mainly Na^+ -channel-poor fibers, were more severely damaged by veratrine. This damage may involve the mobilization of calcium from the sarcoplasmic reticulum and/or mitochondria through an excitation-contraction mechanism stimulated by the alkaloid. The increased cytosolic Ca^{2+} could contribute to cell injury (8), mainly in mitochondria-rich fibers.

Veratrine also changed the proportion of the glycolytic fibers thereby modulating the oxidative and contractile activities. However, there was a significant difference in the direction of this modulation depending on whether the fiber belonged to the SOL or EDL, i.e., the proportion of type I fibers in the SOL decreased by 50%, whereas in the EDL the same fiber type increased in number by 55%. Little is known of how the internal milieu of slow-twitch oxidative muscle (SOL) and fast-twitch glycolytic muscle (EDL) interacts with the toxin to modify the response of the same fiber type to the same stimuli. Similarly, the reason why type I fibers were more susceptible in the SOL muscle and type IIB fibers were more susceptible in EDL is unclear. Melo and Ownby (17) demonstrated that the EDL is more sensitive to the myotoxic action of *Agkistrodon contortrix laticinctus* and *Notechis scutatus scutatus* venoms than SOL muscle. Ownby *et al.* (21) also reported that myotoxin

a, a Na⁺ channel activator from the venom of the North American rattlesnake *Crotalus viridis viridis*, preferentially affected mitochondria-rich (oxidative) fibers rather than mitochondria-poor (glycolytic) fibers. These findings suggest that the susceptibility of a fiber type depends on the myotoxic agent used and on the biochemical, physiological and metabolic properties of the muscle. The present results demonstrate that there are activity-dependent histochemical differences between the responses of predominantly fast-twitch glycolytic muscle (EDL) and slow-twitch oxidative muscle (SOL) to veratrine. They also emphasize the need for determining the type of fibers involved when evaluating the mechanisms underlying the morphological changes induced by myotoxins.

Acknowledgements: The authors thank Dr. Arício X. Linhares for statistical help, Dr. Stephen Hyslop for revising the English, and Mr. Gustavo H. Silva and Mrs. Sueli Michelin for valuable technical assistance. This work was supported by grants from CAPES, CNPq, FAPESP and FAEP-UNICAMP.

REFERENCES

1. Benforado, J. M. 1967. The veratrum alkaloids. In *Physiological pharmacology* (W. S. Root and F. G. Hofmann, Eds.), pp. 331-398. Academic Press, New York.
2. Brooke, M. H. and K. K. Kaiser. 1970. Muscle fiber types: how many and what kind? *Arch. Neurol.* **23**: 369-379.
3. Cameron, D. L. and A. T. Tu. 1978. Chemical and functional homology of myotoxin α from prairie rattlesnake venom and crotamine from South American rattlesnake venom. *Biochim. Biophys. Acta* **532**: 147-154.
4. Catterall, W. A. 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.* **20**: 15-43.
5. Cruz-Höfling, M. A., F. Neder, L. Rodrigues-Simioni and O. Vital Brazil. 1987. Effects of veratrine on the ultrastructure of nerve terminals of the mouse diaphragm *in vitro*. [Abstract] In *Proceedings of the XI Colloquium of the Brazilian Society for Electron Microscopy*, pp. 139. Caxambu, MG, Brasil.
6. Cruz-Höfling, M. A. and O. Vital Brazil. 1990. Alterações ultraestruturais causadas pela veratrina no diafragma isolado de camundongo. [Abstract] In *Proceedings of the V Annual Meeting of FESBE*, pp. 324. Caxambu, MG, Brasil.
7. Davis, C. E., J. B. Harris and L. V. B. Nicholson. 1991. Myosin isoform transitions and physiological properties of regenerated and re-innervated soleus muscles of the rat. *Neuromuscul. Disord.* **1**: 411-421.
8. Duan, C., M. D. Delp, D. A. Hayes, P. D. Delp and R. B. Armstrong. 1990. Rat skeletal muscle mitochondrial $[Ca^{2+}]$ and injury from downhill walking. *J. Appl. Physiol.* **68**: 1241-1251.
9. Dubowitz, V. 1985. Normal muscle. In *Muscle biopsy: a modern approach* (V. Dubowitz, Ed.), pp. 41-81. Bailliere Tindall, London.
10. Engel, W. K. and G. C. Cunningham. 1963. Rapid examination of muscle tissue. An improved trichrome method for fresh-frozen biopsy sections. *Neurology* **13**: 919.

11. Florendo, J. A., J. F. Reger and P. K. Law. 1983. Electrophysiologic differences between mouse *extensor digitorum longus* and *soleus*. *Exp. Neurol.* **82**: 404-412.
12. Guth, L. and F. Samaha. 1969. Qualitative differences between actomyosin ATPase of slow and fast mammalian muscle. *Exp. Neurol.* **25**: 138-152.
13. Honerjäger, P., M. Dugas and X-G. Zong. 1992. Mutually exclusive action of cationic veratridine and cevadine at an intracellular site of cardiac sodium channel. *J. Gen. Physiol.* **99**: 699-720.
14. Jarvis, J. C., T. Mokrusch, M. M. N. Kwende, H. Sutherland and S. Salmons. 1996. Fast-to-slow transformation in stimulated rat muscle. *Muscle Nerve* **19**: 1469-1470.
15. Mattiello-Sverzut, A. C. and M. A. Cruz-Höfling. 2000. Toxin 2 (PhTx 2), a neurotoxic fraction from *Phoneutria nigriventer* spider venom, causes acute morphological changes in mouse skeletal muscle. *Toxicon* **38**: 793-812.
16. Mebs, D. and C. L. Ownby. 1990. Myotoxic components of snake venoms: their biochemical and biological activities. *Pharmacol. Ther.* **48**: 223-236.
17. Melo, P. A. and C. L. Ownby. 1996. Different sensitivity of fast- and slow-twitch muscles to some snake venoms and myotoxins. *Toxicon* **34**: 653-669.
18. Milton, R. L. and M. A. Behforouz. 1995. Na⁺ channel density in extra junctional sarcolemma of fast and slow twitch mouse skeletal muscle fibers: functional implications and plasticity after fast motoneuron transplantation to a slow muscle. *J. Muscle Res. Cell Motil.* **16**: 430-439.
19. Morini, C. C., E. C. L. Pereira, H. S. Selistre de Araújo, C. L. Ownby and T. F. Salvini. 1998. Injury and recovery of fast and slow skeletal muscle fibers affected by ACL myotoxin isolated from *Agkistrodon contortrix laticinctus* (broad-banded copperhead) venom. *Toxicon* **36**: 1007-1024.
20. Nagaoka, R., S. Yamashita, M. Misuno and N. Akaike. 1994. Intracellular Na⁺ and K⁺ shifts induced by contractile activities of rat skeletal muscles. *Comp. Biochem. Physiol.* **109A**: 957-965.
21. Ownby, C. L., D. Cameron and A. T. Tu. 1976. Isolation of myotoxic component from rattlesnake (*Crotalus viridis viridis*) venom. *Am. J. Pathol.* **85**: 149-166.

22. Ownby, C. L., J. M. Gutiérrez, T. R. Colberg, and G. V. Odell. 1982. Quantitation of myonecrosis induced by myotoxin *a* from pairie rattlesnake (*Crotalus viridis viridis*) venom. *Toxicon* **20**: 877-885.
23. Peter, J. B., R. J. Barnard, U. R. Edgerton, C. A. Gillespie, and K. E. Stemple. 1972. Metabolic profiles of three types of fibers of skeletal muscles in guinea pig and rabbits. *Biochemistry* **11**: 2627-2633.
24. Ruff, R. L. 1992. Na⁺ current density at and away from end plates on rat fast- and slow-twitch skeletal muscle fibers. *Am. J. Physiol.* **262**: C229-C234.
25. Salvini, T. F., C. C. Morini, H. S. Selistre de Araújo and C. L. Ownby. 1999. Long-term regeneration of fast and slow murine skeletal muscles after induced injury by ACL myotoxin isolated from *Agkistrodon contortrix laticinctus* (broad-banded copperhead) venom. *Anat. Rec.* **254**: 521-533.
26. Ulbricht, W. 1969. The effect of veratridine on excitable membranes of nerve and muscle. *Ergeb. Physiol. Biol. Exp. Pharmacol.* **61**: 18-71.
27. Ulbricht, W. 1998. Effects of veratridine on sodium currents and fluxes. *Rev. Physiol. Biochem. Pharmacol.* **133**: 1-54.
28. Vital Brazil, O. and M. D. Fontana. 1985. Unequal depolarization of the membrane of the rat diaphragm muscle fibers caused by veratrine. *Pflügers Arch.* **404**: 45-49.
29. Warnick, J. E., E. X. Albuquerque and F. M. Sansone. 1971. The pharmacology of batrachotoxin. I. Effects on the contractile mechanism and on neuromuscular transmission of mammalian skeletal muscle. *J. Pharmacol. Exp. Ther.* **176**: 497-510.
30. Wernig, A., T. F. Salvini and A. Irintchev. 1991. Axonal sprouting and changes in fiber types after running-induced muscle damage. *J. Neurocytol.* **20**: 903-913.

LEGEND FOR FIGURES

Figure 1: Mouse EDL muscle (control): (A) - HE, (B) - NADH-TR reaction showing oxidative fibers (ox), oxidative-glycolytic fibers (og) and glycolytic fibers (gly), (C) and (D) - m-ATPase reaction at pH 4.6 and pH 10.3, respectively (I, IIA and IIB type fibers). Bar = 50 μ m for all panels.

Figure 2: Mouse SOL muscle (control): (A) - H&E, (B) - NADH-TR reaction showing oxidative fibers (ox) and oxidative glycolytic fibers (og), (C) and (D) - m-ATPase reaction at pH 4.6 and pH 10.3, respectively (I and IIA type fibers). Bar = 50 μ m for all panels.

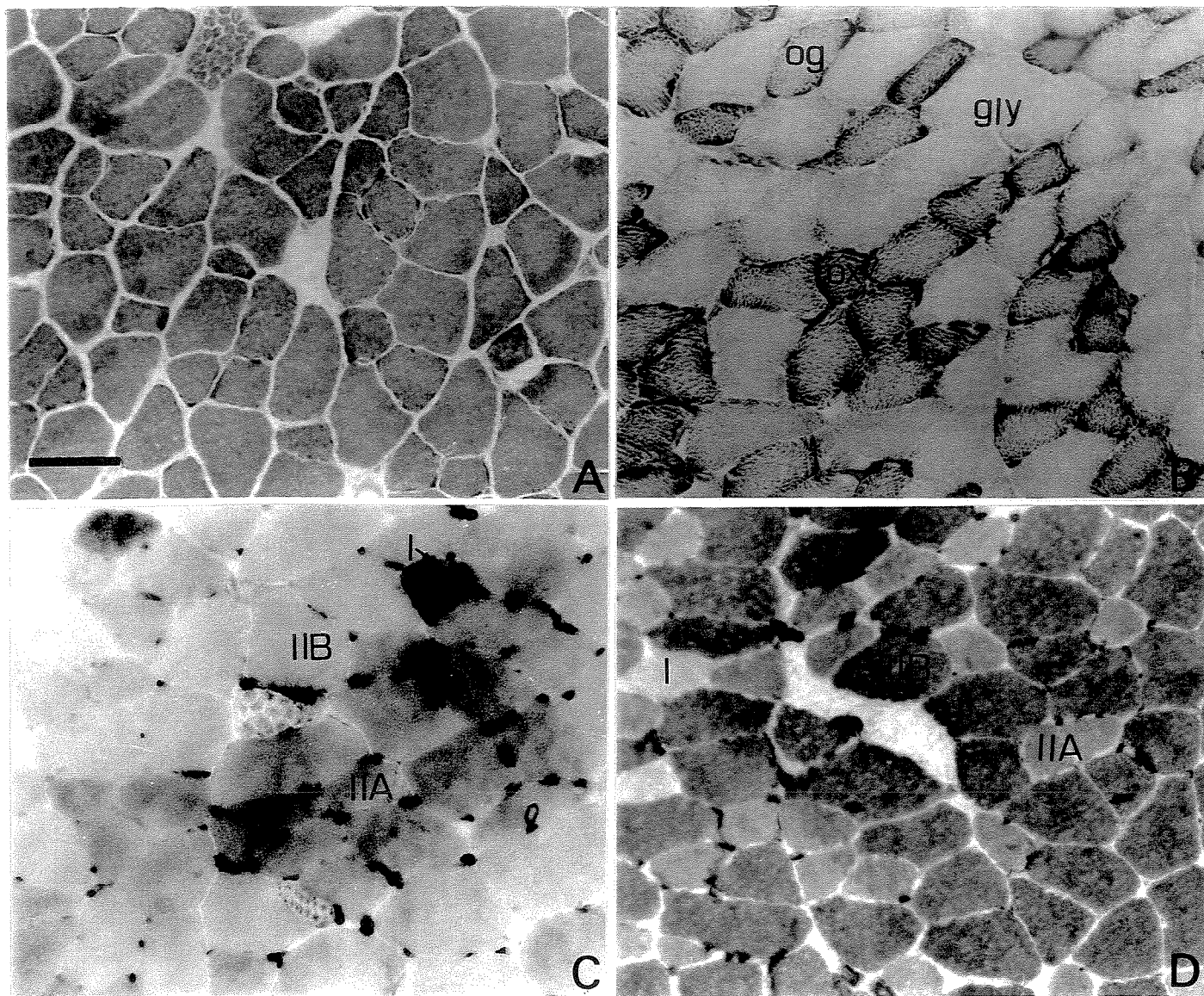
Figure 3: Percentage of fiber types found in the EDL (A) and SOL (B) of saline-treated (control) and veratrine-treated mice. All fibers were typed by the myosin-ATPase.

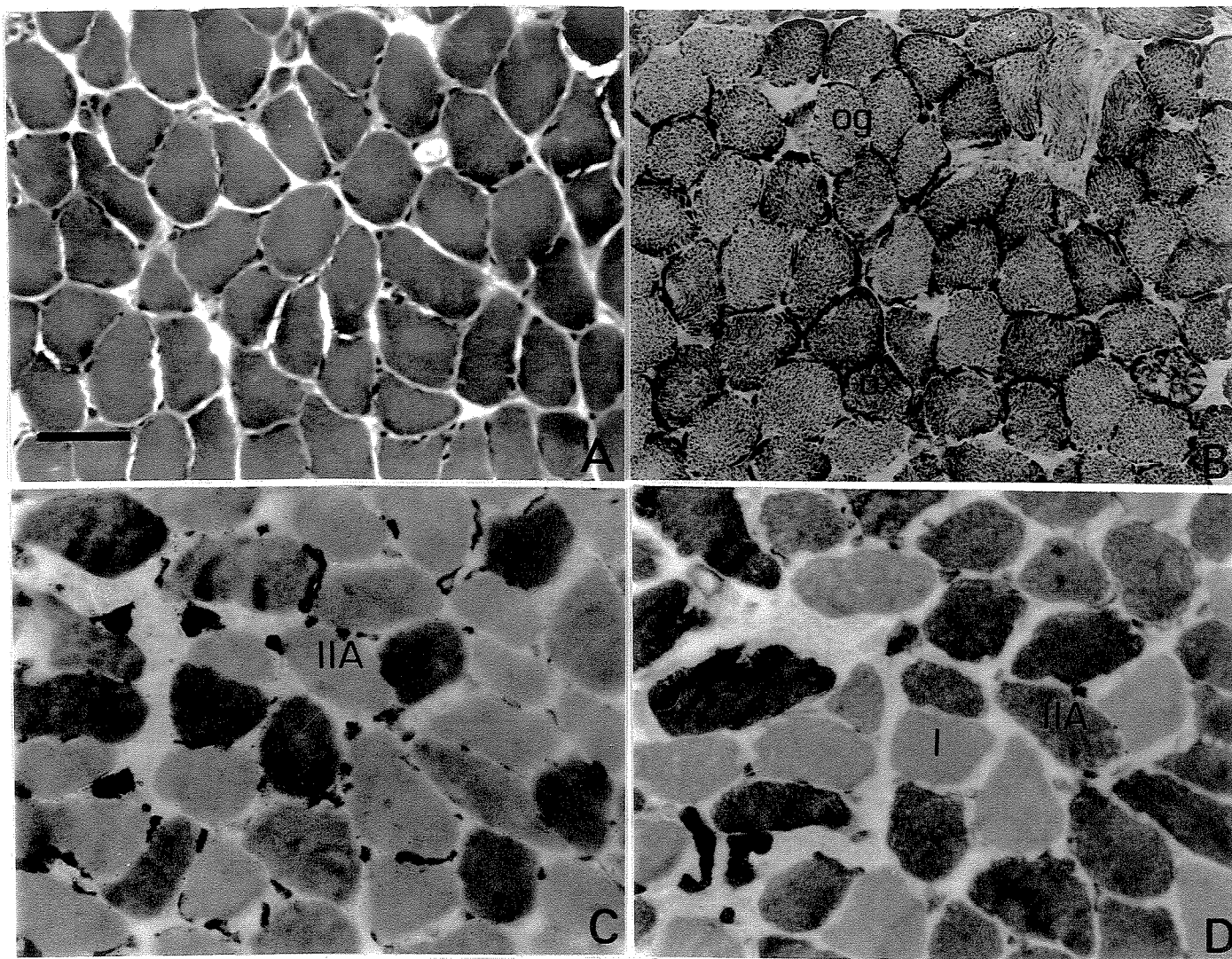
Figure 4: EDL 15 (A and D), 30 (B and E) and 60 min (C and F) after the injection of veratrine. (A) and (C) - HE; (B) Gomori Trichrome staining, (D - F) NADH-TR reaction: note a general decrease in the sarcoplasmic and subsarcolemmal formazan precipitates in all fiber types (ox, og and gly). Arrows indicate the absence or anomalous accumulation of reaction product. h = hypercontracted fibers, dl = delta lesion in a damaged cell, s = swollen cell, (*) = interstitial edema. Bar = 50 μ m for all panels.

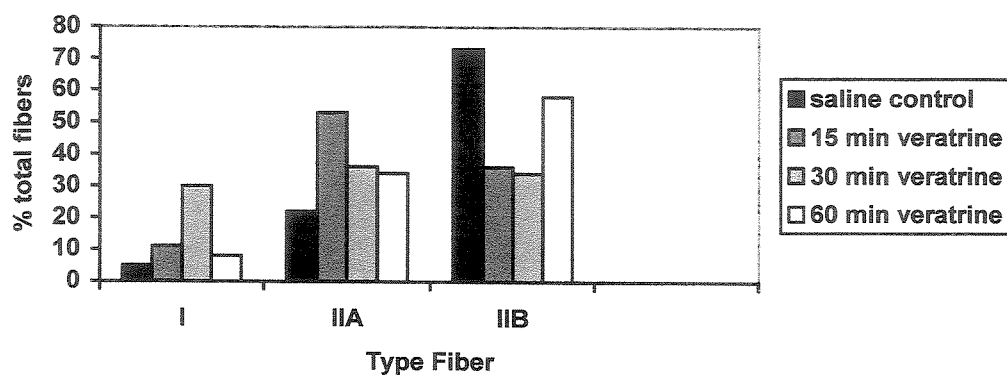
Figure 5: m-ATPase of EDL at 15 (A-B), 30 (C-D) and 60 min (E-F) after the injection of veratrine. Note the fiber types I, IIA and IIB, and abnormally reacting fibers, i.e., fibers which showed no reversal of the reaction (*) after acidic (A, C and E) and alkaline (B, D and F) preincubations. Bar = 50 μ m for all panels.

Figure 6: SOL 15 (A and D), 30 (B and E) and 60 min (C and F) after the injection of veratrine. (A) and (C) - Gomori trichrome staining, (B) - H&E, (D-F) - NADH-TR reaction. The arrows indicate an anomalous or discontinuous accumulation of formazan clusters or deposits in oxidative (ox) and oxidative-glycolytic (og) fibers with an overall decrease relative to the controls. s = swollen and b = highly basophilic cells, fi = fibers with centralized nuclei, c = cell debris, arrowhead = split fibers, (*) = interstitial edema. Bar = 50 μ m for all panels.

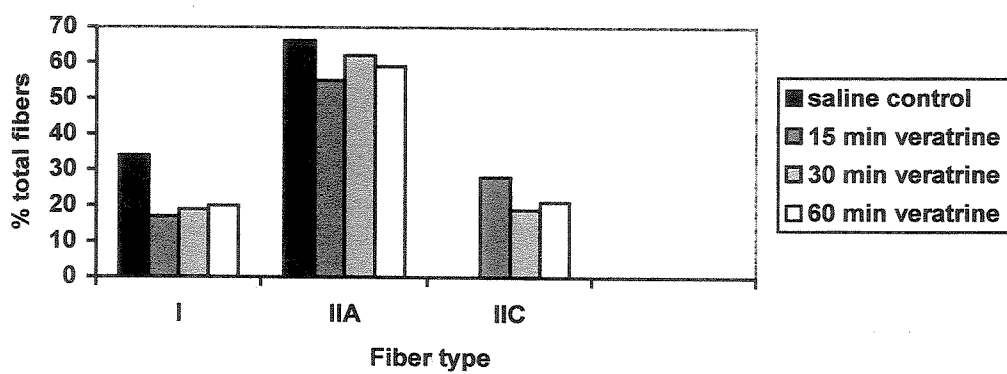
Figure 7: SOL 15 (A-B), 30 (C-D) and 60 min (E-F) after veratrine showing type I and IIA muscle fibers. Atypical fibers which showed no reversal in the reaction under acidic (A, C and E) and alkaline (B, D and F) conditions are also present (*). Bar = 50 μ m for all panels.



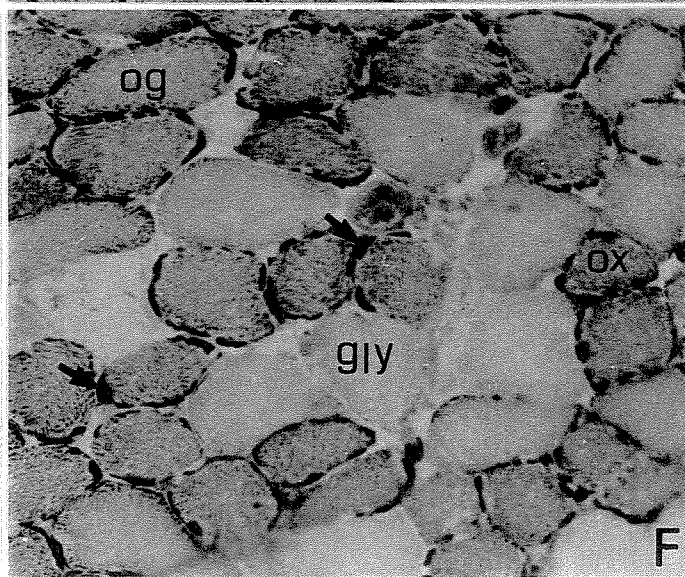
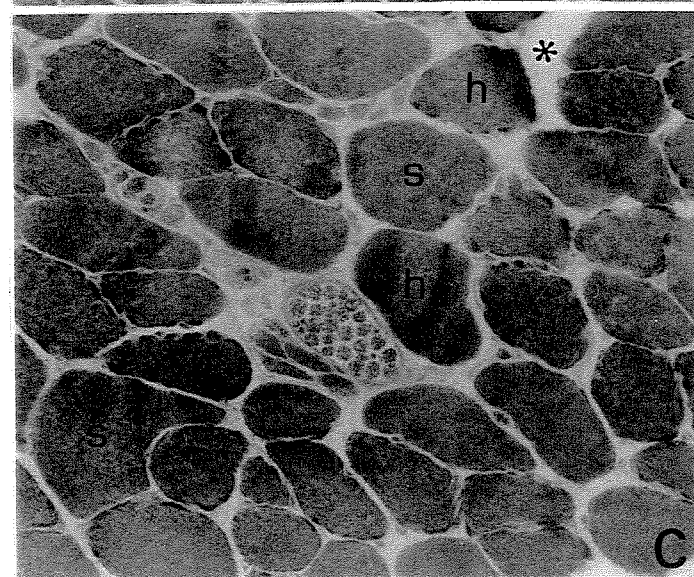
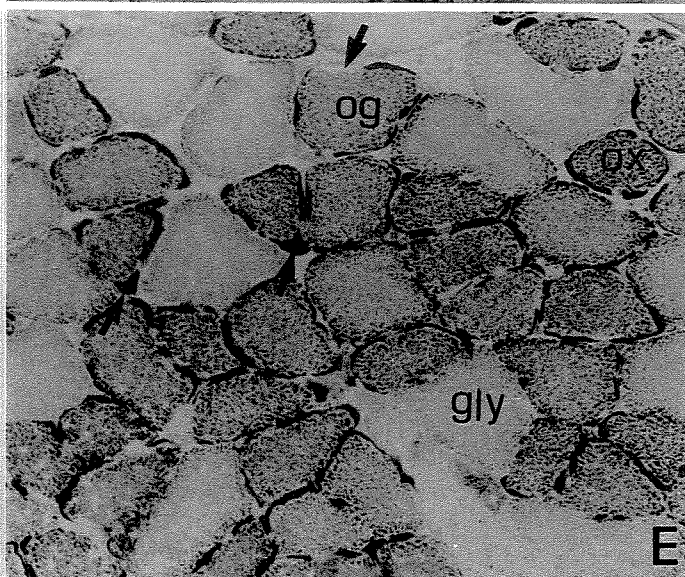
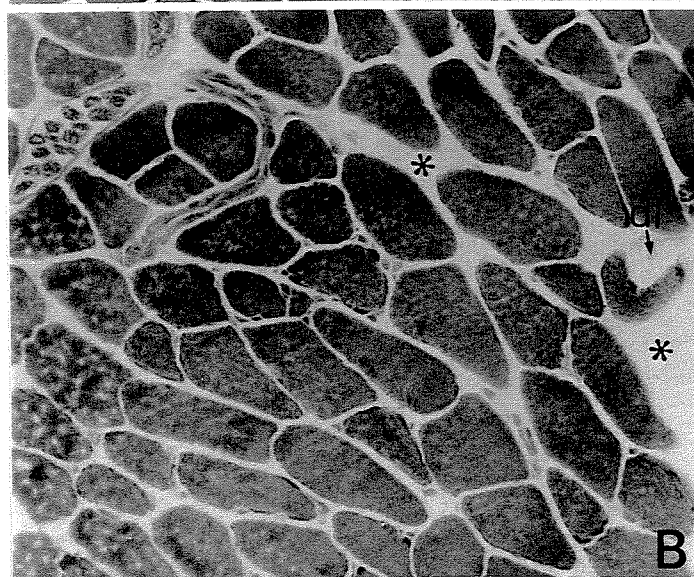
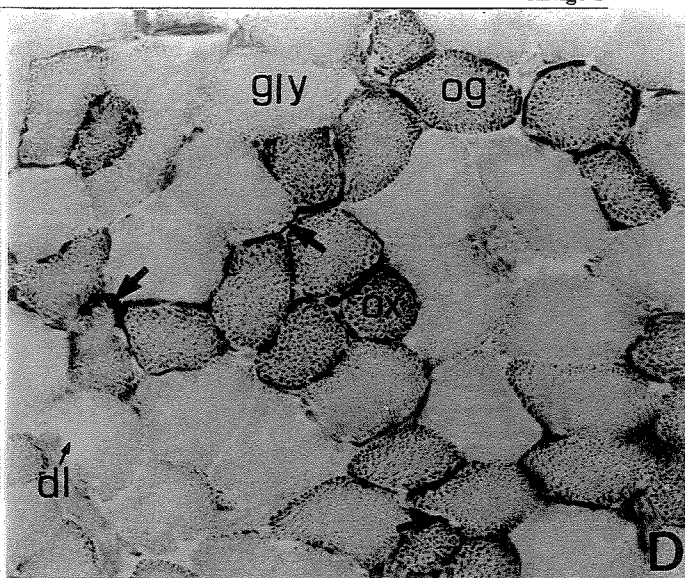
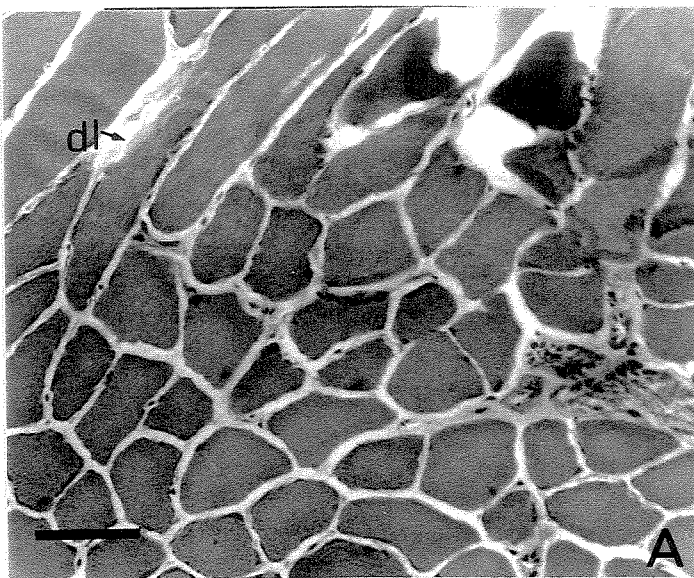


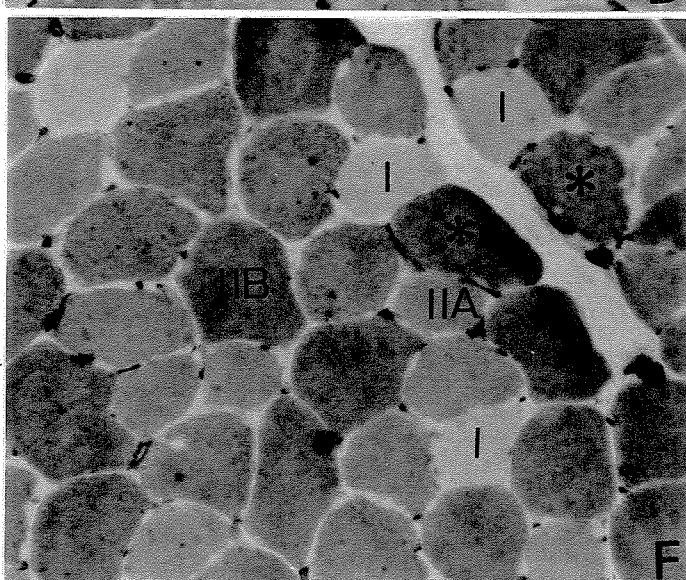
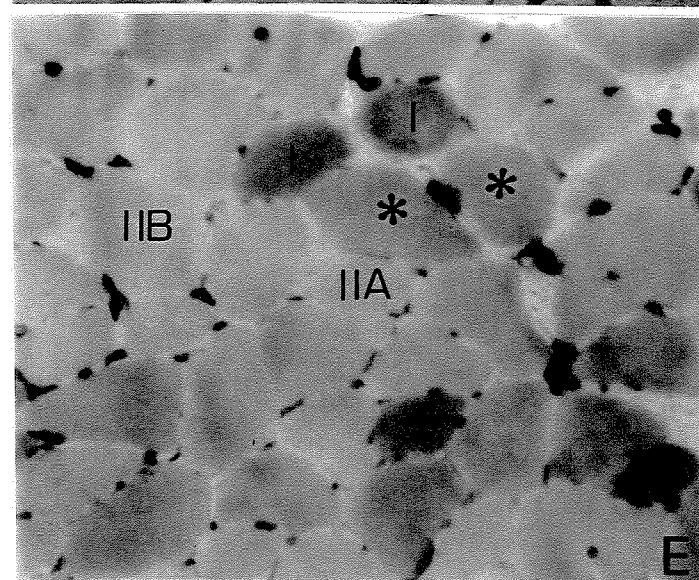
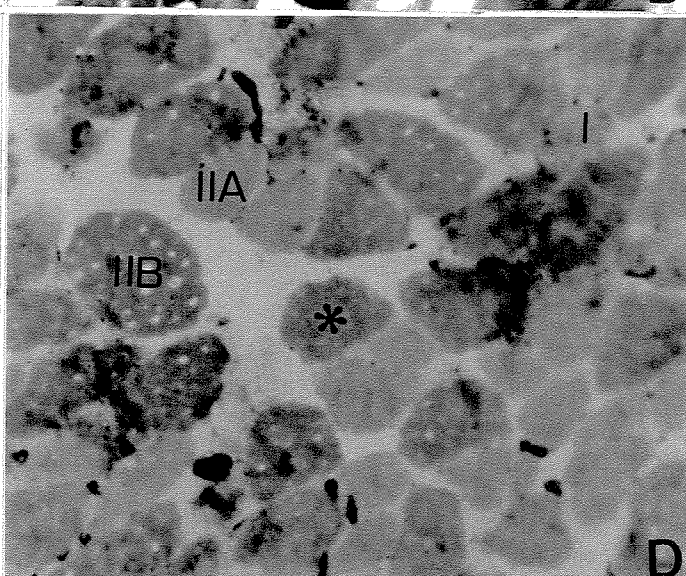
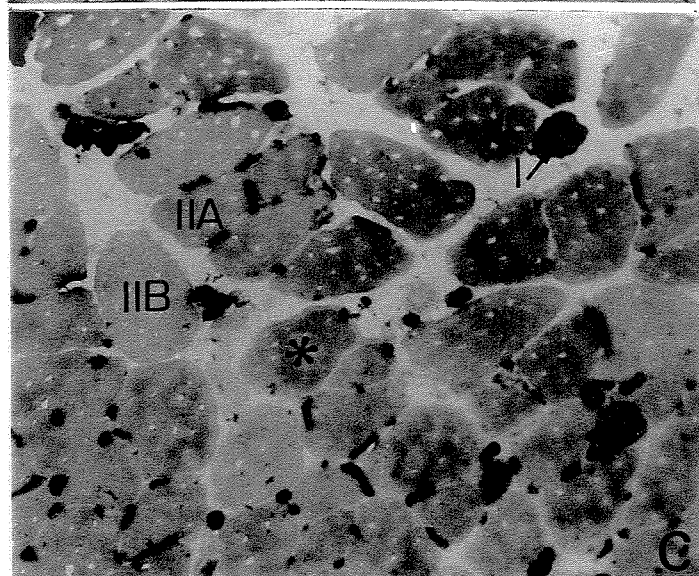
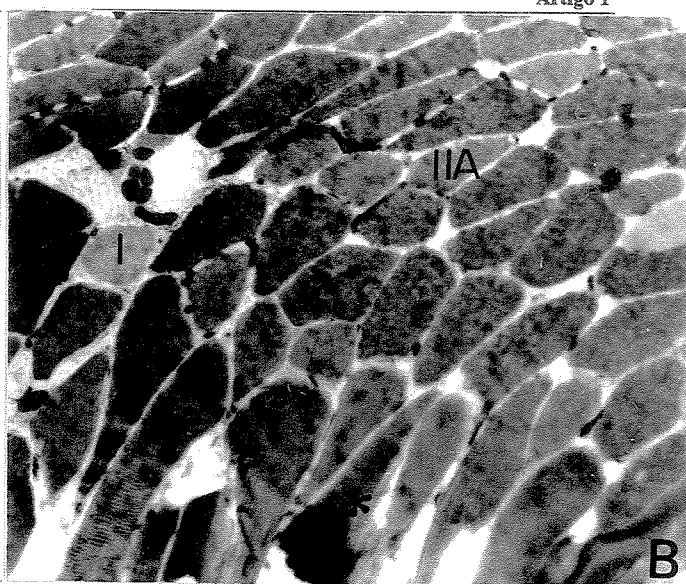
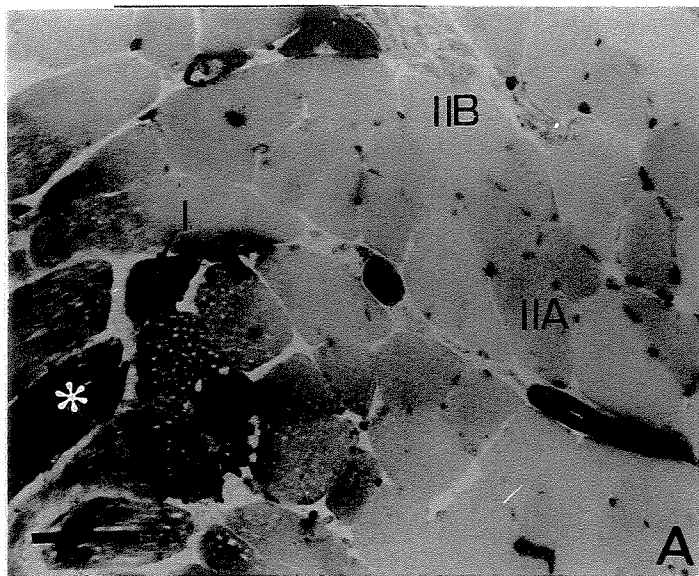


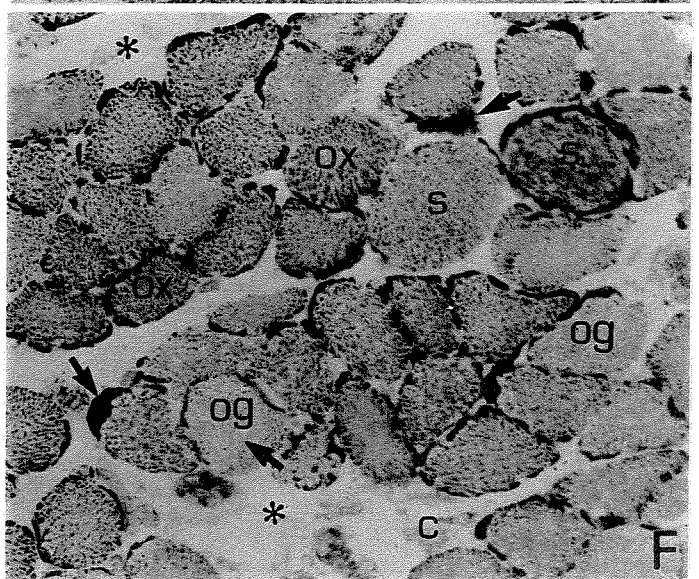
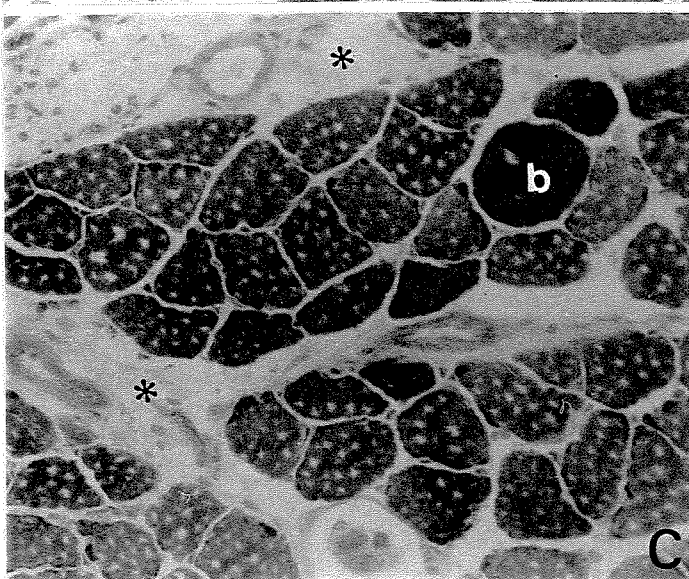
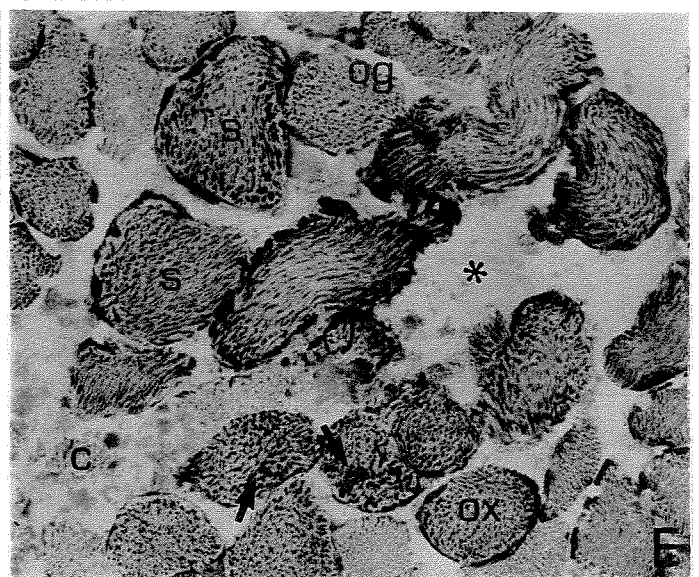
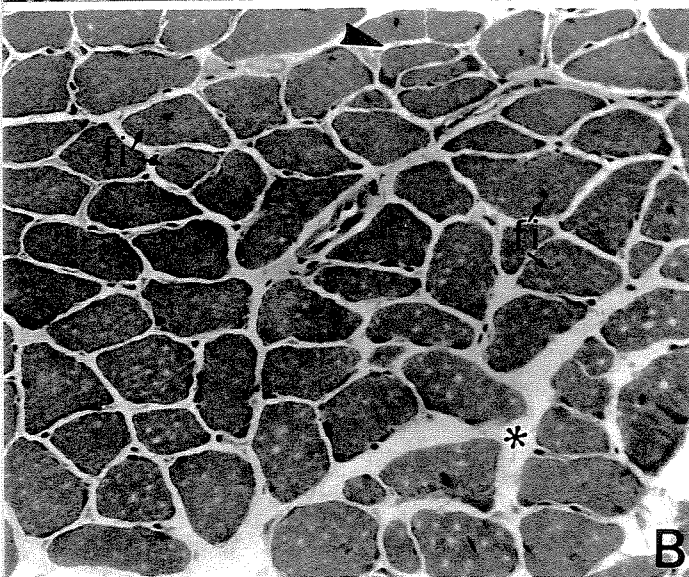
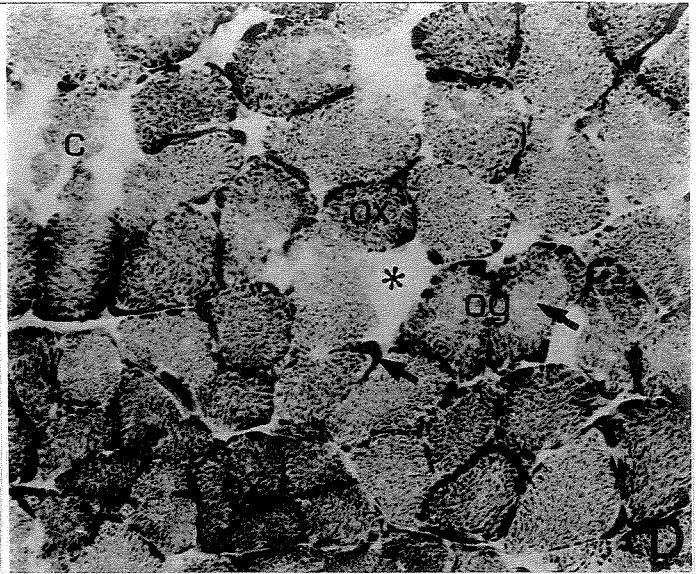
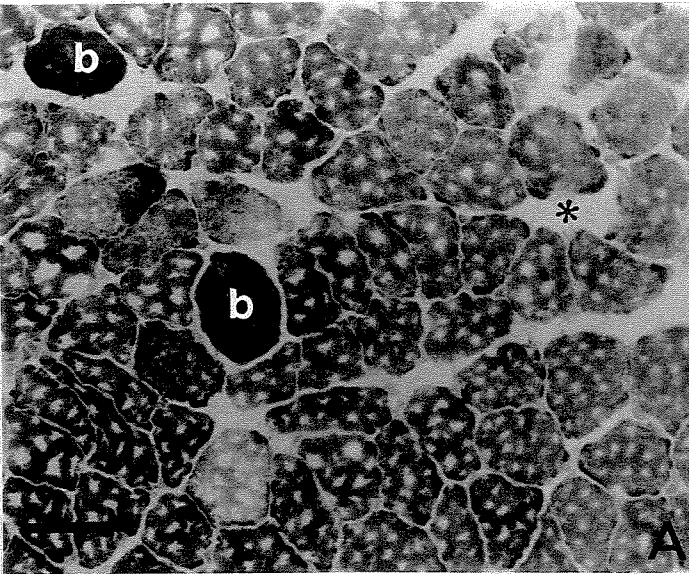
A

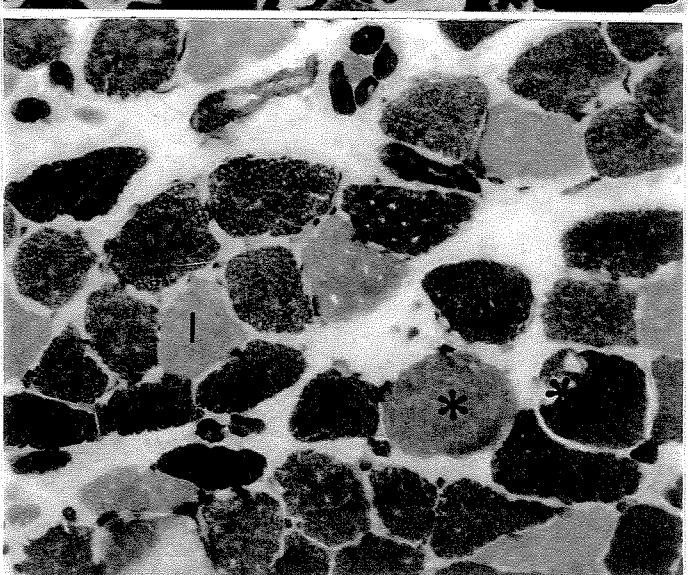
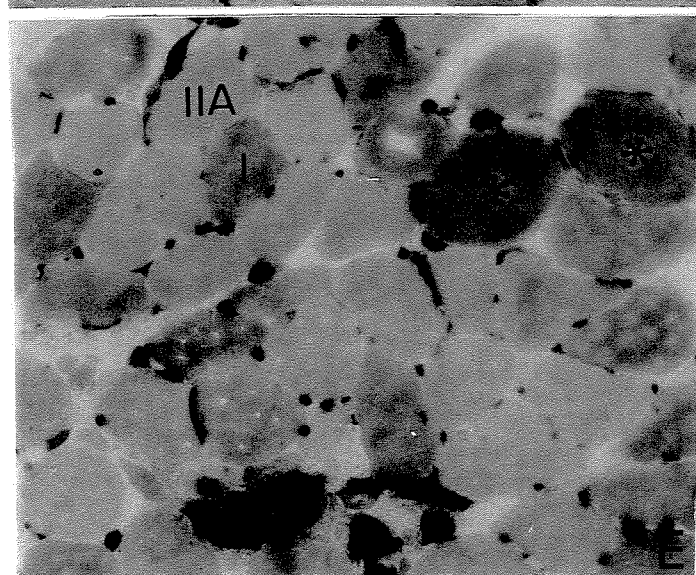
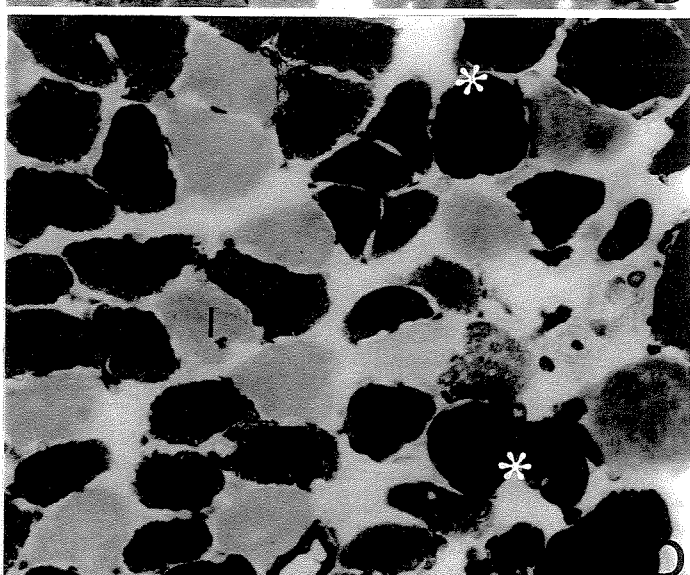
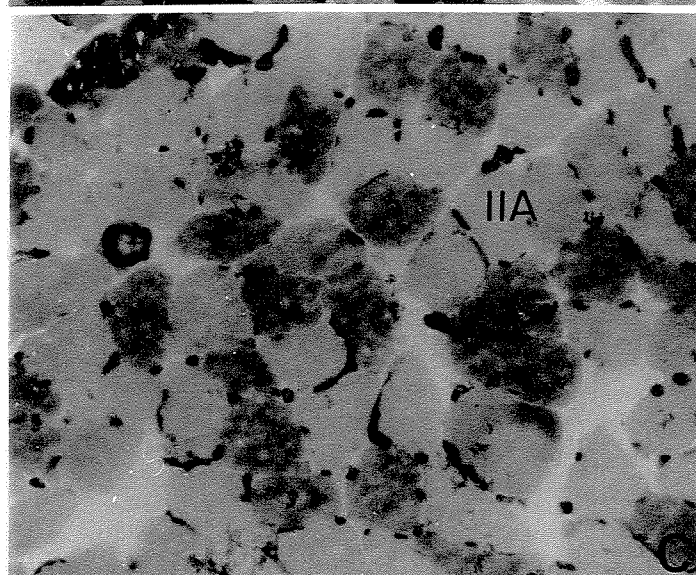
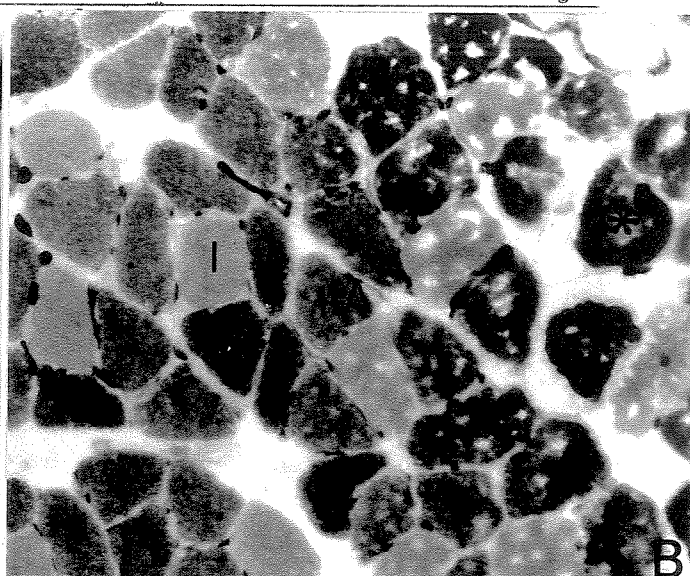
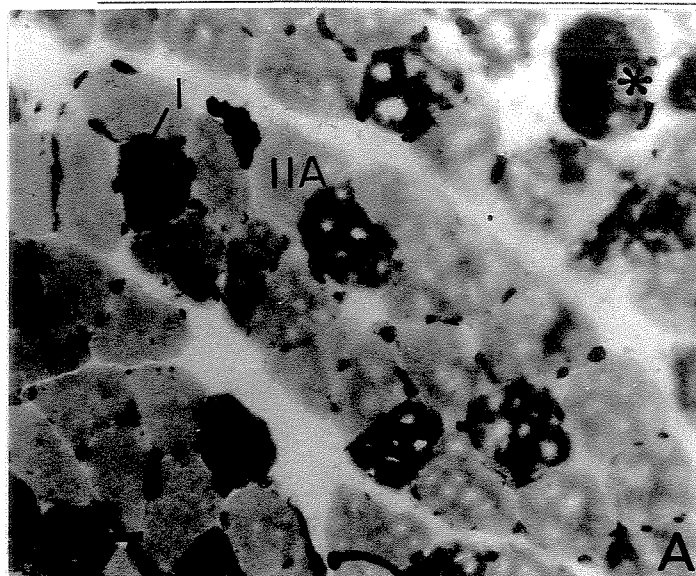


B









✉ Maria Alice da Cruz-Höfling
Departamento de Histologia e Embriologia
Instituto de Biologia , C.P. 6109
Universidade Estadual de Campinas (UNICAMP),
13 087-130, Campinas – SP – BRASIL
email: hofling@obelix.unicamp.br
Tel. (55)(19)3788 7801; Fax. (55)(19)3289 1951

**Mouse *extensor digitorum longus* and *soleus* show distinctive
ultrastructural changes induced by veratrine**

¹Erika Maria Silva Freitas, ²Maeli Dal Pai Silva, ✉¹Maria Alice da Cruz Höfling

¹Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual
de Campinas (UNICAMP), Campinas, SP, ²Departamento de Morfologia, Instituto de
Biociências, Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP), Botucatu,
SP, Brasil

Running title: Veratrine-induced ultrastructural changes in *extensor digitorum longus* and
soleus

ABSTRACT

The ultrastructural effects of veratrine, a Na⁺ channel activator, on the *extensor digitorum longus* (EDL) (fast-twitch) and *soleus* (SOL) (slow-twitch) muscles of the mouse were investigated. Veratrine (5 µl, 10 ng/kg) was injected into the EDL and SOL and the ultrastructural changes were analyzed 15, 30 and 60 min later. The myotoxic effects of the alkaloid in both muscles were compared by analysis of the resulting ultrastructural disturbances. The mitochondria in SOL were affected earlier (within 15 min) than in EDL. Swelling of the terminal cisternae of the sarcoplasmic reticulum was more marked in EDL than in SOL so that fragmentation of the myofilaments was more pronounced in EDL. Hypercontracted sarcomeres were seen mainly in the SOL and veratrine caused infoldings of the sarcolemma only in this muscle. Pyknotic nuclei occurred only in EDL. In both muscles, the T-tubules remained unaffected and by 60 min after veratrine most of the above alterations had reverted to normal. Pretreating the muscles with tetrodotoxin prevented the alterations induced by veratrine. These findings indicated that there were distinctive differences in the morphological effects of veratrine in EDL and SOL. Most of the alterations resulted from the enhanced influx of Na⁺ into muscle fibers, although the entry of veratrine into the cells to act directly on mitochondria cannot be ruled out. These results emphasize the importance of considering the type of muscle when studying the action of myotoxic agents.

Key Words: EDL, mouse, soleus, ultrastructural changes, veratrine

INTRODUCTION

The term veratrum alkaloid is used generically to designate products extracted from species of plants belonging to the family Liliaceae, the best known of which are *Veratrum album*, *V. viride* and *Schoenocaulon officinale* (38). The alkaloid veratrine is a commercial extract from the seeds of *S. officinale* found in South and Central America (23, 39). The extract contains mainly two ester alkaloids, cevadine and veratridine (7) which, together with veratrine, are lipid-soluble activators of voltage-sensitive Na⁺ channels (38) and act by binding reversibly to a hydrophobic site in these channels (5, 39). The resulting increase in sodium permeability is prevented by tetrodotoxin (TTX), a heterocyclic guanidine that blocks Na⁺ channels (10).

Other natural toxins that alter Na⁺-channel kinetics include the alkaloid batrachotoxin from the skin of the frog *Phyllobates aurotaenia* (42), the peptide myotoxin *a* from *Crotalus viridis viridis* rattlesnake venom (33) and the neurotoxic fraction PhTx2 from *Phoneutria nigriventer* armed-spider venom (27). All of these cause morphological changes in skeletal muscle fibers and are considered myotoxins. The myonecrotic effects include fiber vacuolation that results from dilatation of the sarcoplasmic reticulum terminal cisternae without affecting T-tubules. Since these myotoxins generally lack enzymatic activity, only the cell membrane permeability to ions is affected and there is no lysis of the sarcolemma (27, 33, 42).

Experiments *in vitro* (13, 14) have shown that in diaphragm muscle veratrine provokes alterations similar to those described above, and that these changes were abolished by pretreating the preparations with TTX. Veratrine also alters the m-ATPase and NADH-TR activities of the lateral muscle fibers of the freshwater fish *Oreochromis niloticus*, but the structure of the sarcoplasmic reticulum appeared to be unaffected; but strongly affected sarcomeres regular structure; TTX effectively prevented these alterations (Freitas *et al.* unpublished results). The i.m. injection of veratrine in mouse skeletal muscle changed the phenotypic expression of oxidative and glycolytic fibers, altered oxidative and contractile enzyme activities, and produced characteristic cell vacuolation (21). There were also clear differences in the alterations seen in SOL and EDL muscles. These observations

suggested that the effects of veratrine may vary, depending on whether the muscle is of the slow-twitch (SOL) or fast-twitch (EDL) type.

In this work, we used electron microscopy to study the ultrastructural pathological changes induced by veratrine in mouse *extensor digitorum longus* and *soleus* muscles after *in situ* administration of the alkaloid. In some cases, the muscles were pretreated with TTX to determine whether the veratrine-induced alterations were the result of Na⁺ channel activation.

MATERIALS AND METHODS

Adult male mice BALB/c (20-25 g) were obtained from the university's Animal House Unit. A total of 14 animals were used in this study. The mice were housed in standard plastic cages at 25°C on a 12 h light/dark cycle (lights on at 6:00 a.m.) and had access to food and water *ad libitum*. The animals were anesthetized with sodium pentobarbital (40-70 mg/kg) and the *soleus* (SOL) and *extensor digitorum longus* (EDL) muscles were exposed for the injection of veratrine 5 µl (10 ng/kg; Sigma) using a Hamilton syringe. Fifteen, 30 or 60 min later, the anesthetized mice (n=2/group) were perfused through the left ventricle with Karnovsky's fixative (2.5% glutaraldehyde plus 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The muscles were removed and sectioned into small fragments which were then immersed in the same fixative, post-fixed in buffered 1% OsO₄, dehydrated through a graded series of acetone and embedded in an Epon-Araldite resin mixture. Sections (0.5-1 µm thick) were stained with toluidine blue and selected by light microscopy. Ultrathin sections (60-70 nm thick) were stained with lead citrate and uranyl acetate, and examined in a LEO 906 or Philips C-100 transmission electron microscope (TEM). Control groups received the same volume of sterile saline under the identical conditions. In some cases, mice received 1 µl of TTX (50 µM in sterile saline; Sigma), i.m., 5 min before the administration of veratrine. A semi-quantitative approach was used to estimate the extent of the ultrastructural changes in the muscles at each time interval. For this, approximately 20 electron micrographs of each muscle and time were examined in order to establish the effects of veratrine with sarco-tubular system, mitochondria and sarcomeres.

UNICAMP
BIBLIOTECA CENTRAL
SEÇÃO CIRCULANTE

RESULTS

Extensor digitorum longus

Control - Ultrastructural analysis of control EDL showed the typical organization of this muscle, with well-defined sarcomeres and myofibrils aligned with the sarcoplasmic reticulum (SR) terminal cisternae (Fig. 1A).

15-60 min after veratrine injection – Veratrine caused tissue injury at all times up to 60 min. Most fibers were affected. Disorganization of the sarcomeres and poor definition of the Z and M lines and of the A and I bands were the first signs of cell damage as early as 15 min post-injection (Fig. 1B - C). The main change involved dilatation of the sarcoplasmic reticulum (SR) terminal cisternae, which was relatively incipient at 15 min (Fig. 1B - C) and dramatic at 30 min (Fig. 1D). The distortion of the myofilaments seen at 15 min (Fig. 1B) was replaced by extensive damage resulting from stretching and rupture of the myofilaments (Fig. 1D) in many muscle fibers. With the disappearance of the sarcomeres and the rupture of the myofilaments in these severely-injured fibers, rows of vacuoles resulting from the swelling of the SR terminal cisternae were seen aligned in widened sarcoplasmic areas which corresponded to the normally thin intermyofibrillar compartments (Fig. 1D). The mitochondria were intact after 15 min but, after 30 min, were badly-injured and swollen, with several patterns of degeneration involving the cristae and matrix. Pyknotic nuclei were seen in the widened subsarcolemmal space of some cells (Fig. 1E).

By 60 min post-injection, the subsarcolemmal space in some fibers was still abnormally wide (indicating fluid accumulation) but the swelling of the SR cisternae was no longer as extensive as that seen at 30 min (Fig. 2A - B). However, the normal structure of the sarcomeres was lost in an appreciable number of muscle cells, supposedly as a consequence of the mechanical stretching of the myofilaments which were dislocated by the rows of widened SR cisternae. Another prominent finding was the varying thickness of the Z line after 60 min. The T-system remained intact in all cases (Fig. 2A - B). Pretreatment with TTX prevented the veratrine-induced osmotic disturbances (Fig. 2C).

Soleus

Control - The SOL muscle fibers showed regular sarcomeres with well-dimensioned A, H and I bands and clearly visible M and Z lines. Aggregates of normal mitochondria appeared beneath the sarcolemma or between the myofibrils (Fig. 3A).

15-60 min after veratrine injection – Zones of hypercontracted muscle fibers were seen and the bands and lines present in the control sarcomeres now disappeared (Fig. 3B, D and E). The mitochondria also showed a range of degenerative signs involving the cristae and/or the matrix, i.e., swelling, electronlucency, and membrane rupture (Fig. 3C). The onset of these mitochondrial abnormalities was earlier in the SOL (15 min) than in EDL (30 min). However, in both muscles, the morphological signs of an SR hydroelectrolytic imbalance were seen within 15 min. The T-system remained unaffected by veratrine (Fig. 3D - E).

As in EDL, almost all of the signs of severe damage in the SOL disappeared by 60 min, with little or no vacuolation. The sarcolemma showed a sequence of invaginations and the nuclei had extensive folding suggestive of shrinkage and/or of repetitive cycles of contraction (Fig. 4A). Such serial sarcolemmal invaginations were not seen in EDL fibers. After 60 min, the edema persisted beneath the membrane of some cells, but the dimensions of the SR cisternae were generally restored by this time (Fig. 4B). Disrupted myofilaments, a consequence of osmotic-induced changes, were still seen, but were less marked than in EDL (Fig. 4B). As in EDL, TTX completely prevented the veratrine-induced osmotic disturbances (Fig. 4C).

DISCUSSION

Electrophysiological and pharmacological studies with veratrine (40), veratridine (38), batrachotoxin (1) and the highly basic small peptides myotoxin α (25) and crotamine (11) isolated from *Crotalus viridis viridis* and *C. durissus terrificus* rattlesnake venoms, respectively, showed that these substances produced complete depolarization of muscle fibers and increased the frequency and amplitude of end-plate potentials as a result of an increase in Na^+ conductance. The structural damage produced by these toxins in skeletal muscle fibers was in keeping with the physiological findings.

Ultrastructural analysis showed that veratrine produced short-lived necrosis in EDL and SOL which resembled that seen in mouse isolated diaphragm preparations incubated with this same compound (14). The myonecrosis was characterized by swelling of the SR terminal cisternae, disorganization of the sarcomeres with splitting and/or rupture of the myofilaments, Z-line destructuring, hypercontraction of the sarcomeres that lead to myofilament clumping, and marked mitochondrial damage. However, the effects of veratrine on the ultrastructure of SOL and EDL muscles varied. Although both muscles showed a similar onset (15 min) in SR terminal cisternae dilatation, by 30 min the dilatation of the SR in EDL had progressed faster and showed more severe pathological effects than in SOL. This difference was reflected in the extensive myofilament disruption in the EDL at 60 min, even after the regression of vacuolation.

EDL and SOL have phenotypic, functional, biochemical and metabolic differences that are ultimately determined by the type of motor innervation (31). Thus, fast-contracting glycolytic muscles have a higher number of T-tubules (15, 17), a higher frequency of miniature end-plate potentials which means a greater number of acetylcholine (ACh) molecules combining with ACh receptors per unit time (20), a less developed vascular supply (2), a well-developed sarcotubular system, fewer mitochondria accumulated between the sarcolemma and myofibrils, thinner Z lines (32), and a higher density of Na^+ channels in the neuromuscular junctions (29, 36), which allows a higher influx of Na^+ during contractile activity (30) than occurs in slow-contracting oxidative muscles. These characteristics of fast-contracting glycolytic muscles probably contribute to the greater effects of veratrine seen in the SR of EDL.

The SR disturbances resulted from Na^+ channel activation since they were prevented by TTX. Although it is still unclear which ions accumulate in the SR cisternae to promote the entrance of water, the most likely candidate is Ca^{2+} . Impairment of the Na^+ - Ca^{2+} exchanger caused by the accumulation of Na^+ in the cytosol may be one of the mechanisms involved. The Na^+ - Ca^{2+} exchanger is a non ATP-dependent pump that, under normal physiological conditions, transports three Na^+ ions into the cell for each Ca^{2+} ion pumped out (4, 8). This exchanger is widely distributed in the sarcolemma of cardiac cells, smooth muscle cells and neural tissue (43), but also occurs in intact single skeletal muscle fibers of the frog (9, 22) and mouse (3), and in isolated T-tubule membranes of the frog skeletal muscle (16).

During cardiac cell Na^+ overload, the Na^+ - Ca^{2+} exchanger operates in a reverse mode, i.e., one Ca^{2+} ion enters the cell for every three Na^+ pumped out, thus triggering Ca^{2+} release from the sarcoplasmic reticulum (26, 43). A similar or identical mechanism could account for the SR swelling seen after veratrine. This mechanism would involve a higher influx of Na^+ into the sarcoplasm to promote the activation of Ca^{2+} -ATPase and the capture of cytosolic Ca^{2+} by SR, followed by the passive release of Ca^{2+} by the SR. Stimulation of the Ca^{2+} -ATPase and an increase in SR Ca^{2+} loading has been reported for myotoxin *a* in isolated rabbit skeletal muscle SR (41). In this case, myotoxin *a* bound to the SR membranes and uncoupled Ca^{2+} uptake from Ca^{2+} -dependent ATP hydrolysis. A greater uptake of Ca^{2+} by SR cisternae through a Ca^{2+} -ATPase could increase the permeability of the SR membrane to anions and water, leading to cisternal dilatation (18) or spontaneous Ca^{2+} release (6). An increase in Ca^{2+} release from the SR can also occur through the activation of intracellular ryanodine-sensitive Ca^{2+} channels, as seen with some peptide myotoxins, and myotoxins with PLA_2 activity (*cf.* 19).

An increase in cytosolic Ca^{2+} could also result from the repetitive depolarization of fibers by veratrine. Veratridine decreases the replenishment of ACh in synaptic vesicles from the electric organ of *Torpedo californica* and elicits a sustained depolarization of the neuromuscular junction in rat phrenic nerve-diaphragm preparations. The latter action activates voltage-sensitive Ca^{2+} channels, which in turn increases the miniature end-plate potential and miniature end plate currents (35). In fast contracting muscle, the repetitive depolarization of fibers quickly exhausts the ACh stores of end-plate vesicles, and these are

not replenished in the presence of the alkaloid. Nerve terminals depleted of synaptic vesicles have been observed in mouse phrenic nerve-diaphragm preparations incubated with veratrine (10 µg/ml) for 60 min (14).

We have recently observed that veratrine rapidly increases oxidative enzyme NADH-TR reactions in type I, IIA and IIB muscle fibers and alters the contractile activity of these fibers in lateral muscle of the fish *Oreochromis niloticus* (Freitas *et al.* unpublished results). Veratrine also affects the histochemistry of SOL and EDL muscle fibers in the mouse (21). In SOL, veratrine significantly decreased the number of type I fibers, but maintained the number of type IIA fibers. Conversely, in EDL, veratrine significantly increased the number of type I fibers and decreased those of type IIB. These observations suggested that veratrine interacted differently with each muscle, and that the response of the same muscle fiber type (slow-twitch or fast-twitch) varied depending on the fibers' location (SOL or EDL). These findings also suggested that iterative conditions (muscle *vs* internal tissue characteristics *vs* gene expression) might control (or modulate) the fiber phenotype. A direct action of veratrine on mitochondria could be responsible for the greater effect of this alkaloid in SOL. Indeed, veratrine uncouples respiration in mitochondria isolated from the muscle of rats and mice by non-specifically increasing in the permeability of the inner mitochondrial membrane to protons (unpublished observations).

The possibility that substances that act on Na⁺ channels can also enter cells to act intracellularly has been suggested by several authors (11, 12, 25, 27, 28, 37, 41) to explain the SR swelling seen after alterations in Na⁺ conductance. Tu and Morita (37) reported that peroxidase-conjugated myotoxin *a* bound to the SR membranes of human skeletal muscle *in vitro*, but did not examine whether this also occurred *in vivo*. Mebs and Ownby (28) suggested that myotoxin *a* crossed the plasma membrane or gained access to the SR via the T-tubules. Like other Na⁺ channel-activating toxins such as batrachotoxin, myotoxin *a*, and toxin PhTx2 from spider *P. nigriventer* venom, veratrine has no enzymatic activity and is not cytolytic so is unable to damage the cell membrane and reach the sarcoplasm. Some myotoxins, generally those with PLA₂ activity or a cytolytic action, induce myonecrosis by activating tissue lipases (19), but this seems an unlikely mechanism for veratrine.

The hydrophobic nature of veratrine most likely facilitates its interaction with the corresponding hydrophobic region of the Na⁺ channel (5). Honerjäger *et al.* (24) showed

that veratridine interacts with the protonated form of site 2 of the Na^+ channel and gains access to the cytosol by diffusing through the membrane in its uncharged form. Should such internalization also occur with veratrine, it would help to explain the stronger effect seen in SOL muscle which is more dependent on oxidative metabolism and has a higher number of mitochondria.

These results indicate that the ultrastructural effects of veratrine in EDL and SOL are mediated by a direct action on Na^+ channels since TTX effectively abolished the abnormalities. The opening of Na^+ channels by veratrine probably leads to an electrolytic imbalance within the cell which, in turn, could account for the alterations seen in the SR and mitochondria. It is also possible that veratrine may cross the cell membrane to act directly on intracellular organelles. The differences in the ultrastructural effects of veratrine in SOL and EDL probably reflect the phenotypic, physiological and metabolic characteristics of these muscles. Such differences need to be considered when investigating the myotoxic action of any substance.

Acknowledgments: The authors thank Gustavo H. da Silva, Maria Helena Moreno and Luciana P. Le Sueur for technical assistance, and Dr. Stephen Hyslop for revising the English. E.M.S.F. is the recipient of a scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). This work was supported by grants from FAPESP, CNPq and FAEP/UNICAMP.

REFERENCES

1. Albuquerque, E. X., J. E. Warnick and F. M. Sansone. 1971. The pharmacology of batrachotoxin. II. Effect on electrical properties of the mammalian nerve and skeletal muscle membranes. *J. Pharmacol. Exp. Ther.* **176**: 511-528.
2. Appell, H. J. 1984. Variability in microvascular pattern dependent upon muscle fiber composition. In *Skeletal muscle microcirculation*. (F. Hammersen and K. Messmer, Eds.), p. 15. Karger, Basel.
3. Balnave, C. D. and D. G. Allen. 1998. Evidence for $\text{Na}^+/\text{Ca}^{2+}$ exchange in intact single skeletal muscle fibers from the mouse. *Am. J. Physiol.* **274**: C940-C946.
4. Barcenas-Ruiz, L., D. J. Beuckelman and W. G. Wier. 1987. Sodium-calcium exchange in heart: membrane currents and changes in $[\text{Ca}^{2+}]$. *Science* **238**: 1720-1722.
5. Becker, S. and R. D. Gordon. 1992. Sodium channel specific neurotoxins: recent advances in the understanding of their molecular mechanisms. In *Handbook of Experimental Pharmacology. Selective Neurotoxicity*, vol. 102. (H. Herken and F. Hucho, Eds.), pp. 719-737. Springer-Verlag, New York-Berlin.
6. Beeler, T. 1983. Osmotic changes of sarcoplasmic reticulum vesicles during Ca^{2+} uptake. *J. Memb. Biol.* **76**: 165-171.
7. Benforado, J. M. 1967. The veratrum alkaloids. In *Physiological pharmacology* (W. S. Root and F. G. Hofmann, Eds.), pp. 331-398. Academic Press, New York.
8. Blaustein, M. P. 1974. The interrelationship between sodium and calcium fluxes across cell membranes. *Rev. Physiol. Biochem. Pharmacol.* **70**: 32-82.
9. Castillo, E., H. Gonzalez-Serratos, H. Rasgado-Flores and M. Rozycka. 1991. $\text{Na}^+-\text{Ca}^{2+}$ exchange studies in frog phasic muscle cells. *Ann. N.Y. Acad. Sci.* **639**: 554-557.
10. Catterall, W. A. 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.* **20**: 15-43.
11. Chang, C. C. and H. K. Tseng. 1978. Effect of crotamine, a toxin of South American rattlesnake venom, on the sodium channel of murine skeletal muscle. *Br. J. Pharmacol.* **63**: 551-559.

12. Chang, C. C., S. J. Hong and M. J. Su. 1983. A study on the membrane depolarization of skeletal muscles caused by a scorpion toxin, sea anemone toxin II and crotoxin and the interaction between toxins. *Br. J. Pharmacol.* 79: 673-680.
13. Cruz-Höfling, M. A., F. Neder, L. Rodrigues-Simioni and O. Vital Brazil. 1987. Effects of veratrine on the ultrastructure of nerve terminals of the mouse diaphragm *in vitro*. [Abstract] In *Proceedings of the XI Colloquium of the Brazilian Society for Electron Microscopy*, pp. 139. Caxambu, MG, Brasil.
14. Cruz-Höfling, M. A. and O. Vital Brazil. 1990. Alterações ultraestruturais causadas pela veratrina no diafragma isolado de camundongo. [Abstract] In *Proceedings of the V Annual Meeting of FESBE*, pp. 324. Caxambu, MG, Brasil.
15. Dauber, W. 1979. Zur Fasertypischen Morphologie und Funktion der Triaden im Skelettmuskel des Frosches (*Rana sculentia*). *Z. Microsc. Anat. Forsch.* 93: 512-536.
16. Donoso, P. and C. Hidalgo. 1989. Sodium-calcium exchange in transverse tubules isolated from frog skeletal muscle. *Biochim Biophys Acta* 978: 8-16.
17. Eastwood, A. B., C. Franzini-Armstrong and C. Peracchia. 1982. Structure of membranes in crayfish muscle: comparison of phasic and tonic fibers. *J. Muscle Res. Cell Motil.* 3: 273-294.
18. Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* 57: 71-108.
19. Fletcher, J. E., M. Hubert, S. J. Wieland, Q-H. Gong and M-S. Jiang. 1996. Similarities and differences in mechanisms of cardiotoxins, melittin and other myotoxins. *Toxicon* 34: 1301-1311.
20. Florendo, J. A., J. F. Reger and P. K. Law. 1983. Electrophysiologic differences between mouse *extensor digitorum longus* and *soleus*. *Exp. Neurol.* 82: 404-412.
21. Freitas, E. M. S., M. Silva-Dal Pai and M. A. Cruz-Höfling. 2000. A histochemical study of the interaction of mouse *extensor digitorum longus* and *soleus* muscles with veratrine. *Exp. Neurol.* (submitted).
22. García, M. C., A. F. Díaz, R. Godínez and J. A. Sánchez. 1992. Effect of sodium deprivation on contraction and charge movement in frog skeletal muscle fibers. *J. Muscle Res. Cell Motil.* 13: 354-365.

23. Honerjäger, P. 1982. Cardioactive substances that prolong the open state of sodium channels. *Rev. Physiol. Biochem. Pharmacol.* **92**: 1-74.
24. Honerjäger, P., M. Dugas and X-G. Zong. 1992. Mutually exclusive action of cationic veratridine and cevadine at an intracellular site of cardiac sodium channel. *J. Gen. Physiol.* **99**: 699-720.
25. Hong, S. J. and C. C. Chang. 1985. Electrophysiological studies of myotoxin α , isolated from prairie rattlesnake (*Crotalus viridis viridis*) venom, on murine skeletal muscles. *Toxicon* **23**: 927-937.
26. Levesque, P. C., N. Leblanc and J. R. Hume. 1994. Release of calcium from guinea pig cardiac sarcoplasmic reticulum induced by sodium-calcium exchange. *Cardiovasc. Res.* **28**: 370-378.
27. Mattiello-Sverzut, A. C. and M. A. Cruz-Höfling. 2000. Toxin 2 (PhTx 2), a neurotoxic fraction from *Phoneutria nigriventer* spider venom, causes acute morphological changes in mouse skeletal muscle. *Toxicon* **38**: 793-812.
28. Mebs, D. and C. L. Ownby. 1990. Myotoxic components of snake venoms: their biochemical and biological activities. *Pharmacol. Ther.* **48**: 223-236.
29. Milton, R. L. and M. A. Behforouz. 1995. Na^+ channel density in extra junctional sarcolemma of fast and slow twitch mouse skeletal muscle fibers: functional implications and plasticity after fast motoneuron transplantation to a slow muscle. *J. Muscle Res. Cell Motil.* **16**: 430-439.
30. Nagaoka, R., S. Yamashita, M. Misuno and N. Akaike. 1994. Intracellular Na^+ and K^+ shifts induced by contractile activities of rat skeletal muscles. *Comp. Biochem. Physiol.* **109A**: 957-965.
31. Nemeth, P. M., D. Petter and G. Vrbová. 1981. Comparison of enzyme activities among single muscle fibres within defined motor units. *J. Physiol.* **311**: 489-495.
32. Ogata, T. 1988. Morphological and cytochemical features of fiber types in vertebrate skeletal muscle. *CRC Crit. Rev. Anat. Cell Biol.* **1**: 229-275.
33. Ownby, C. L., D. Cameron and A. T. Tu. 1976. Isolation of a myotoxic component from rattlesnake (*Crotalus viridis viridis*) venom. *Am. J. Pathol.* **85**: 149-166.

34. Pellegrini Filho, A., O. Vital Brazil, M. D. Fontana. 1978. The action of crotonamine on skeletal muscle: an electrophysiological study. In *Toxins: Animal, Plant and Microbial* (P. Rosenberg, Ed.), pp. 375-382. Pergamon Press, Oxford.
35. Pemberton, K. E., M. L. Nguyen, C. Prior, S. Parsons and I. G. Marshall. 1995. Effect of veratridine on miniature endplate current amplitudes at the rat neuromuscular junction and acetylcholine uptake by *Torpedo* synaptic vesicles. *Brain Res.* 671: 267-274.
36. Ruff, R. L. 1992. Na⁺ current density at and away from end plates on rat fast- and slow-twitch skeletal muscle fibers. *Am. J. Physiol.* 262: C229-C234.
37. Tu, A. T. and M. Morita. 1983. Attachment of rattlesnake venom myotoxin α to sarcoplasmic reticulum: peroxidase conjugated method. *Br. J. Exp. Pathol.* 64: 633-637.
38. Ulbricht, W. 1969. The effect of veratridine on excitable membranes of nerve and muscle. *Ergeb. Physiol. Biol. Exp. Pharmacol.* 61: 18-71.
39. Ulbricht, W. 1998. Effects of veratridine on sodium currents and fluxes. *Rev. Physiol. Biochem. Pharmacol.* 133: 1-54.
40. Vital Brazil, O. and M. D. Fontana. 1985. Unequal depolarization of the membrane of the rat diaphragm muscle fibers caused by veratrine. *Pflügers Arch.* 404: 45-49.
41. Volpe, P., E. Damiani, A. Maurer and A. T. Tu. 1986. Interaction of myotoxin α with the Ca²⁺-ATPase of skeletal muscle sarcoplasmic reticulum. *Archs. Biochem. Biophys.* 246: 90-97.
42. Warnick, J. E., E. X. Albuquerque and F. M. Sansone. 1971. The pharmacology of batrachotoxin. I. Effects on the contractile mechanism and on neuromuscular transmission of mammalian skeletal muscle. *J. Pharmacol. Exp. Ther.* 176: 497-510.
43. Yashar, P. R., M. Fransua and W. H. Frishman. 1998. The sodium-calcium ion membrane exchanger: physiologic significance and pharmacologic implications. *J. Clin. Pharmacol.* 38: 393-401.

LEGEND FOR FIGURES

Figure 1- *Electron micrographs of EDL muscle fibers (longitudinal sections).*

(A) - Control: Normal fiber morphology. Bar = 0.5 μm ; (B-C) - Fifteen minutes after veratrine: note the loss of the regular organization of the sarcomeres, and the numerous vacuoles resulting from SR swelling. T = intact T-tubules, m = myelin figures, mi = mitochondria. Bars = 0.5 μm ; (D-E) - Thirty minutes after veratrine. (D) shows a severely-affected cell with a massive loss of myofilaments and numerous membranous profiles resulting from SR cisternae dilatation. (D-E), mi = mitochondria with vesicular cristae or tightly juxtaposed cristae. N = pyknotic nucleus, T = intact T-tubules. Bars = 1 μm .

Figure 2 - *Electron micrographs of mouse EDL 60 min after veratrine (A-B) or after treatment with TTX followed by veratrine (C).*

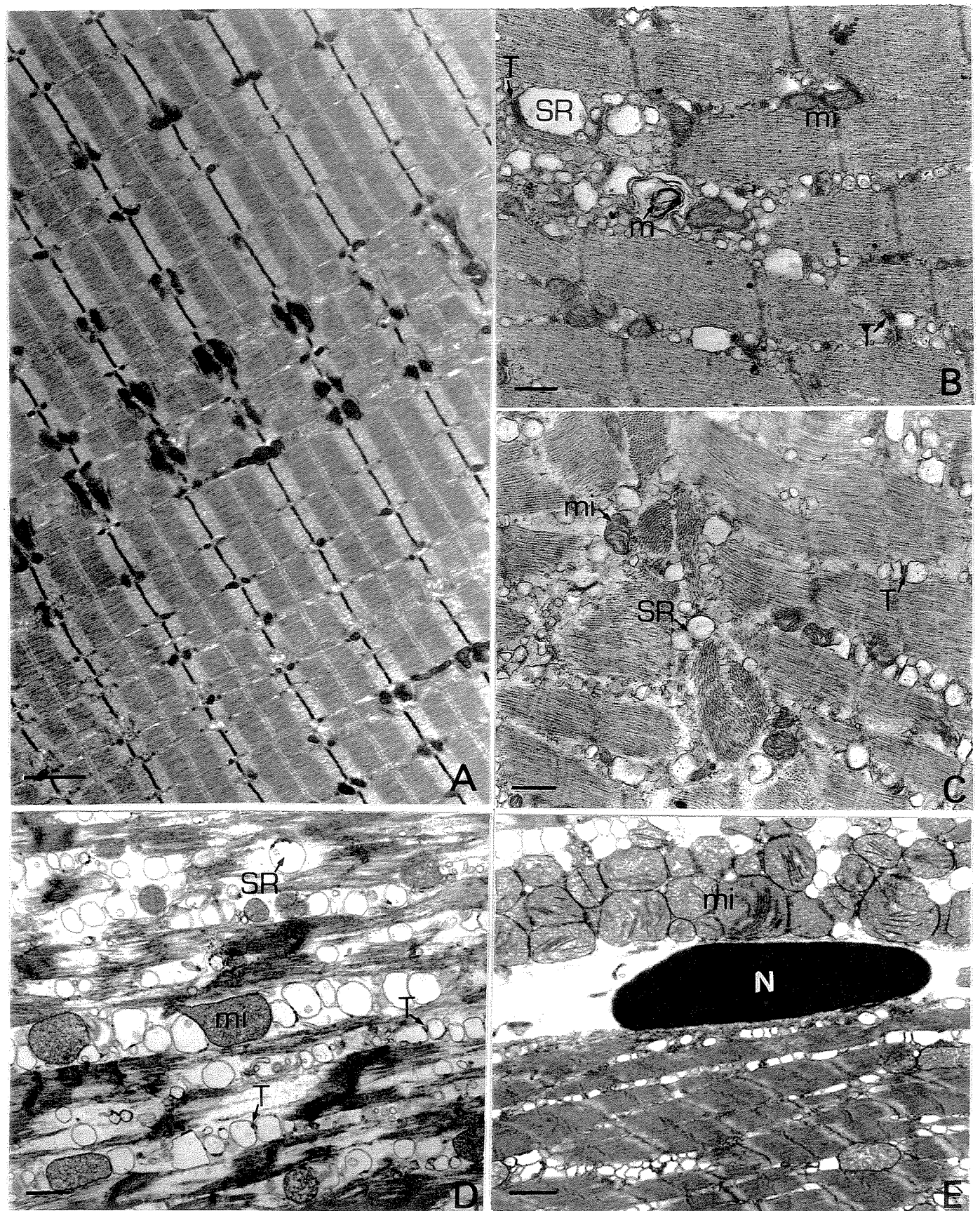
(A-B) - Affected muscle cells showing abnormal Z line and irregular sarcomeres. No SR vacuoles are seen. (*) edema. mi = highly damaged mitochondria. m = myelin figure; (C) - Detail of a cell showing the protection of the SR system. T = intact T-tubules in the middle of normal terminal cisternae. Bars = 1 μm , 0.5 μm and 0.125 μm for A, B and C, respectively.

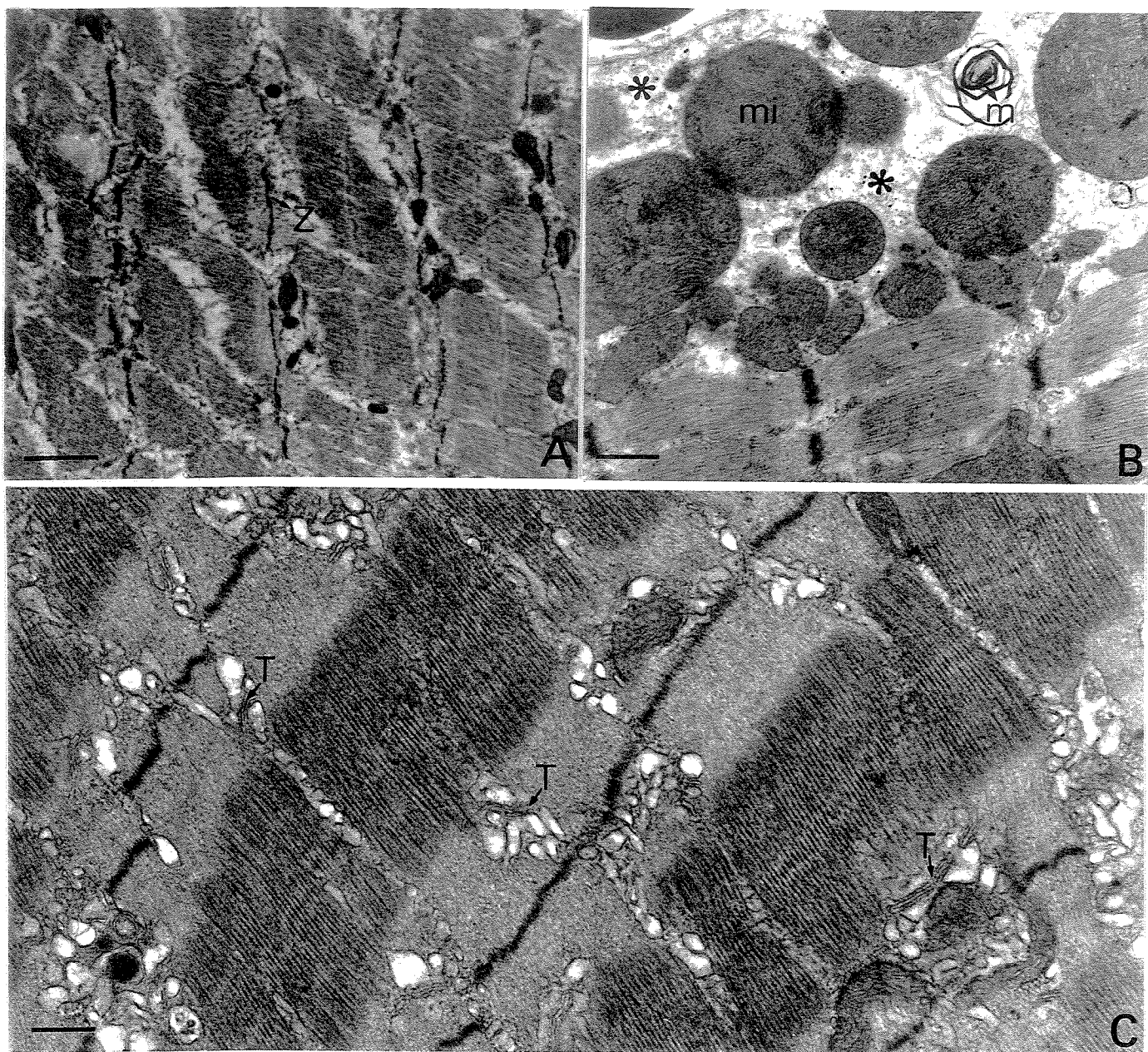
Figure 3 - *Electron micrographs of SOL muscle fibers (longitudinal sections)*

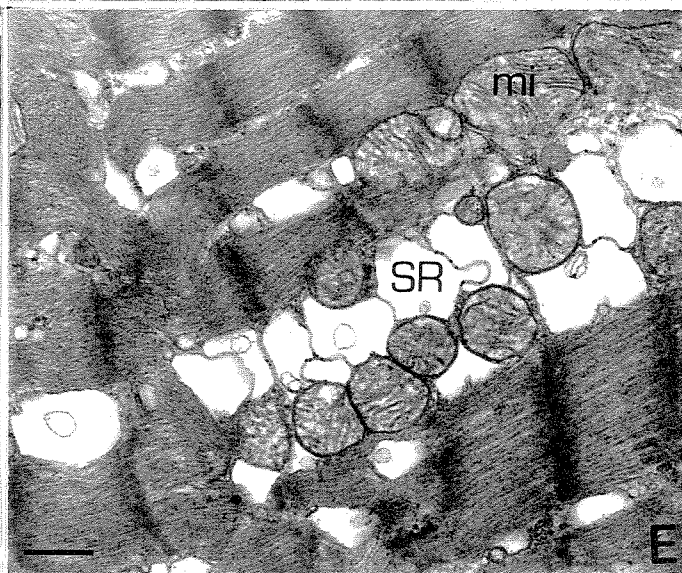
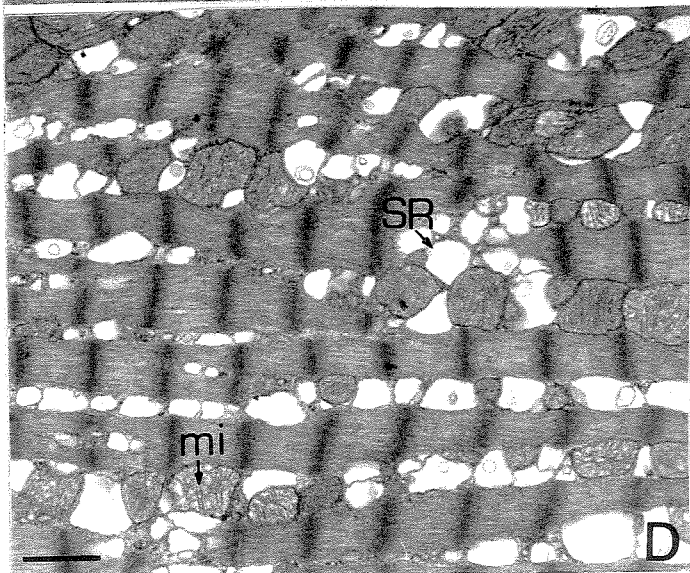
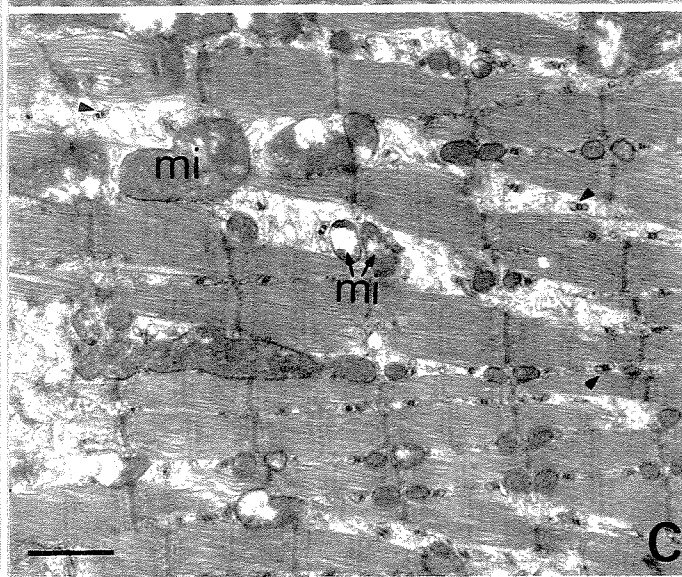
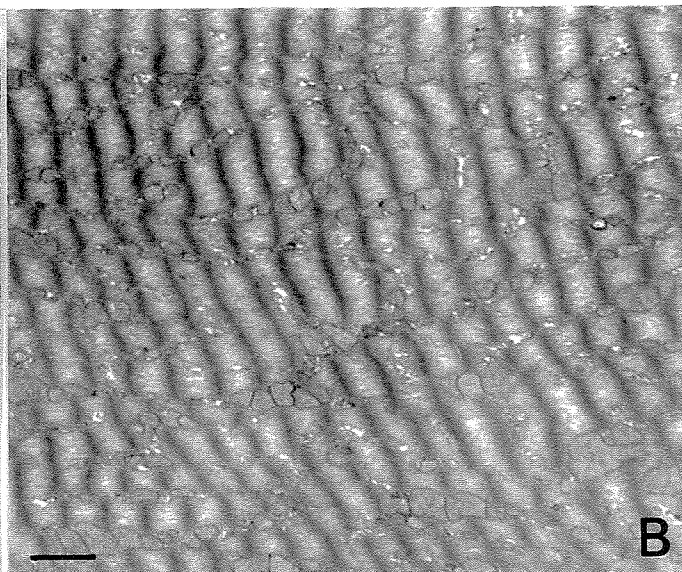
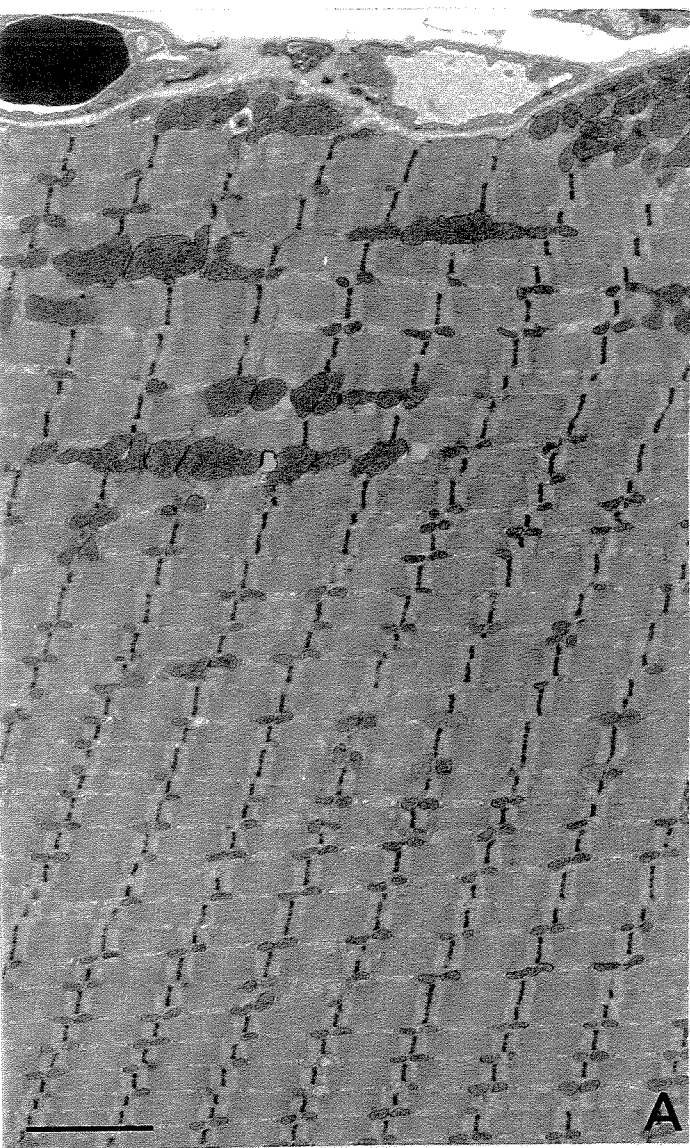
(A) - Normal appearance of oxidative muscle. Bar = 1 μm ; (B-C) - Two pathological states seen after 15 min veratrine treatment: hypercontracted sarcomeres (B), and highly degenerated mitochondria (mi) (C) - Note that in the latter case, the sarcotubular system was undisturbed (arrowheads). Bars = 1 μm ; (D-E) - Thirty min after veratrine showing hypercontracted sarcomeres and rows of enlarged SR terminal cisternae. mi = mitochondria with electronlucent matrix and diffuse cristae. Bars = 1 μm and 0.5 μm for D and E, respectively.

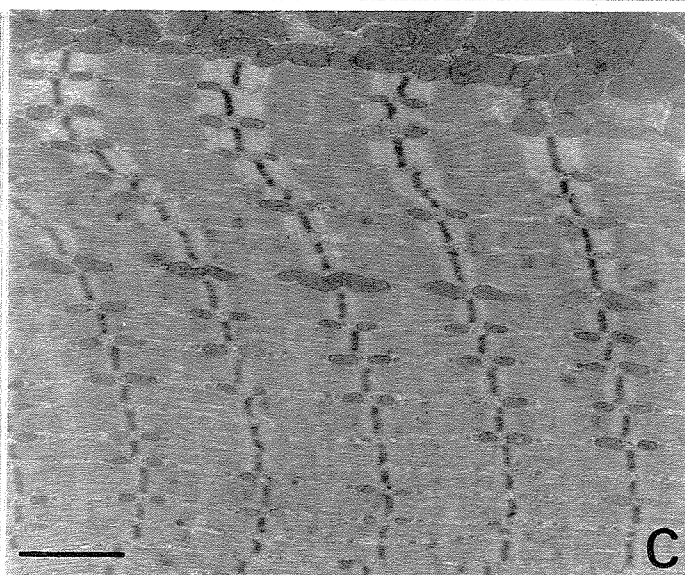
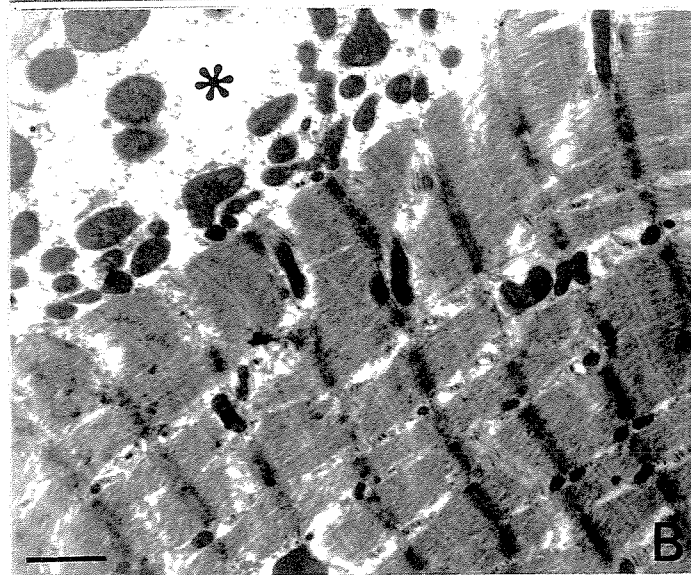
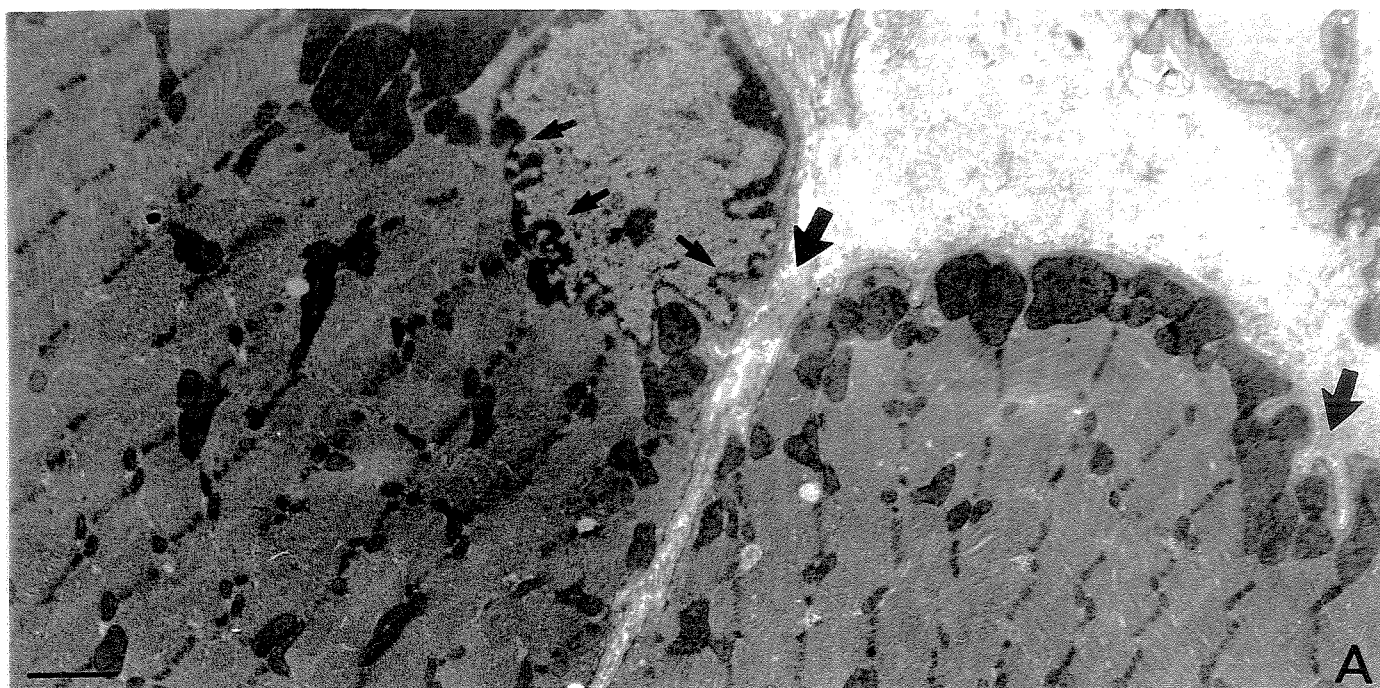
Figure 4 - *Electron micrographs of SOL after veratrine and TTX + veratrine.*

(A-B) - By 60 min after veratrine, the normal morphology was generally restored, but deep infoldings were seen in the sarcolemma (large arrows) and the outline of the nucleus was corrugated (small arrows); the mitochondria had a normal morphology; (B) - Detail showing a damaged fiber 60 min after veratrine. (*) edema; (C) - With TTX, the abnormal features induced by veratrine did not occur. Bars = 1 μm , 1 μm and 0.5 μm for A, B and C, respectively.









✉ Maria Alice da Cruz-Höfling
Departamento de Histologia e Embriologia
Instituto de Biologia – C.P. 6109
Universidade Estadual de Campinas
13 087-130 Campinas – SP – BRASIL
email: hofling@obelix.unicamp.br
Tel. (55)(19)3788 7801; Fax. (55)(19)3289 195

**HISTOENZYMOLOGICAL AND ULTRASTRUCTURAL CHANGES
IN LATERAL MUSCLE FIBERS OF *OREOCHROMIS NILOTICUS*
(TELEOSTEI: CICHLIDAE) AFTER LOCAL INJECTION OF
VERATRINE**

¹Erika Maria Silva Freitas, ²Maeli Dal Pai Silva, ✉¹Maria Alice da Cruz-Höfling

¹Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, ²Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP), Botucatu, SP, Brasil

Running title: Veratrine-induced changes in skeletal muscle of *O. niloticus*

ABSTRACT

The effects of veratrine, an alkaloid mixture extracted from the plant *Schoenocaulon officinale*, were studied in the lateral muscle of the freshwater teleost *Oreochromis niloticus* after intramuscular injection. Histoenzymological typing of the muscle fibers before and 15, 30 and 60 min after the injection of veratrine (10 ng/kg fish) was used to assess the morphological changes. Electron microscopy was also used to evaluate the ultrastructural alterations caused by the alkaloid. In some cases, muscles were pretreated with tetrodotoxin to determine whether the changes were the result of Na⁺ channel disturbances. Veratrine altered the metabolism of fibers mainly after 30 min. Oxidative fibers showed decreased activity, whereas that of glycolytic and oxidative-glycolytic type fibers was increased. There was no change in the m-ATPase activity of the three fiber types, except at 60 min post-veratrine, when a novel fiber type, which showed no reversal after acidic and alkaline preincubations, appeared. Ultrastructural analysis showed that veratrine-induced myonecrosis was characterized by disorganization of the sarcomeres, dissociation and/or rupture of the myofilaments and degeneration of the mitochondria. Dilatation of the sarcoplasmic reticulum terminal cisternae was discrete, and the T-tubules remained intact. Veratrine caused a time-dependent increase in the length of lateral muscle sarcomeres, as shown by transmission electron microscopy, in contrast to the hypercontracted fibers seen with light microscopy. Pretreatment with tetrodotoxin (1 ng/ml) prevented the ultrastructural changes caused by veratrine.

Key words: fish, histoenzymology, muscle, ultrastructural changes, veratrine

INTRODUCTION

Veratrine, an alkaloid extracted from the plant *Schoenocaulon officinale* and other species of the family Liliaceae (Ulbricht, 1998), belongs to a heterogeneous group of natural substances, including many of animal origin, which interfere with the physiology of voltage-dependent Na^+ channels. In mammalian muscle, veratrine causes repetitive firing and depolarization of excitable membranes as a result of increased sodium permeability (Benforado, 1967; Vital Brazil and Fontana, 1985). Morphologically, veratrine causes vacuolation in muscle fibers and hypertrophy of sarcoplasmic reticulum terminal cisternae with no change in the T-system of the phrenic nerve diaphragm preparation. In addition, nerve endings are depleted of synaptic vesicles and the axoplasm becomes swollen and electronlucent (Cruz-Höfling and Vital Brazil, 1990). *In vivo*, veratrine alters the phenotypic profile in muscle fibers of mouse *extensor digitorum longus* (EDL) and *soleus* muscles as demonstrated by the NADH-TR and m-ATPase reactions (Freitas et al., 2000a). Pharmacologically, veratrine prolongs the action potential and increases in Na^+ and Ca^{2+} influx (Sutro, 1986; Lehoueller et al., 1988; Nánási et al., 1994) in invertebrate and frog skeletal muscle. As a result, the inactivation of Na^+ channels is delayed (Ulbricht, 1998). In this study, we examined the ultrastructural changes in the muscle fibers of the lateral musculature of the freshwater teleost *Oreochromis niloticus* (Nile tilapia) following exposure to veratrine. Histoenzymological techniques were used to determine whether veratrine affected the expression of slow-twitch oxidative (type I), fast-twitch glycolytic oxidative (type IIA) and fast-twitch glycolytic (type IIB) fibers.

MATERIALS AND METHODS

Juvenile specimens of *Oreochromis niloticus* (Teleostei: Cichlidae) 12 cm long and weighing 25-35 g were obtained from CEPTA, Pirassununga, SP, Brazil. The fish were caught during the spring and kept in suitable tanks until used. One group of fish (n=9) received 5 µl of veratrine intramuscular (i.m.) (10 ng/kg) in the lateral musculature after anesthesia with 3-aminobenzoic acid ethyl (MS 222 - 1:100000), while another group (n=6) received 1 µl of TTX (1 ng/ml, Sigma) i.m. 5 min before the administration of veratrine. Fifteen, 30 or 60 min later, the fish were sacrificed by cervical dislocation and the muscle excised. Control fish (n=9) received an injection of sterile saline under the same experimental conditions.

Histoenzymological studies

Small fragments of lateral muscle were immediately frozen in liquid N₂ and stored at -80°C. Serial frozen sections (8 µm thick) were obtained with a cryostat and stained with hematoxylin-eosin (HE), or Gomori's trichrome for morphological examination. Some sections were also stained for nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR; Peter et al., 1972) and myosin ATPase (m-ATPase) activity after acidic (pH 4.6) or alkaline (pH 10.2) preincubations (Guth and Samaha, 1969; Brooke and Kaiser, 1970) to allow histochemical typing of the fibers. A pH range of 4.3 - 4.6 and 10.1 - 10.3 was tested for acidic and alkaline preincubations in order to establish the best conditions for m-ATPase activity.

Ultrastructural studies

At specific times after injection, the lateral muscle was quickly removed and sectioned into small fragments, which were immediately immersed in Karnovsky solution (2.5 % glutaraldehyde plus 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4), post-fixed in 1% OsO₄, dehydrated through graded acetone and embedded in an Epon-Araldite mixture. Sections (0.5 - 1 µm thick) were stained with toluidine blue and examined by light

microscopy. Ultrathin sections (60-70 nm thick) stained with lead citrate and uranyl acetate were examined in a LEO 906 or Philips C-100 transmission electron microscope (TEM).

Chemicals

All reagents were from Sigma Chemical Co. (St. Louis, MO, USA), with the exception of glutaraldehyde which was from Polysciences, Inc. (Warrington, PA, USA).

RESULTS

Morphology and histochemistry

Saline-treated fish: - The lateral musculature of saline-injected *O. niloticus* was organized in more or less parallel bundles of fibers in which small-diameter “red” fibers were arranged as a superficial triangular mass, while large “white” fibers, which were heterogeneous in size, were located at a deeper position (Fig. 1A). The white fibers were polygonal and outnumbered the red fibers. A further group of morphologically and biochemically heterogeneous fibers formed an intermediate zone between the superficial and deep layers (Fig. 1A). The red muscle fibers showed high oxidative enzyme activity by the NADH-TR reaction in the subsarcolemmal and intermyofibrillar region (Fig. 1B), as well as acidic-stable and alkaline-labile m-ATPase activities, characteristic of type I fibers (Fig. 1C). The white fibers showed moderate to weak oxidative activity in the NADH-TR reaction (Fig. 1B) and strong to moderate alkaline-stable and acidic-labile m-ATPase activities (Fig. 1C). Fibers with these activities were classified as fast-twitch oxidative glycolytic (type IIA) and glycolytic (type IIB) fibers. After acidic preincubation, the fibers of the intermediate zone, disposed as a mosaic, showed strong, moderate and weak m-ATPase reactions corresponded to type I, IIA and IIB fibers, respectively (Fig. 1D). Small and large fibers in this muscle layer showed similar patterns of m-ATPase activity.

Veratrine-treated fish: - Veratrine did not change the distribution of muscle fiber types in the three zones seen in control fish. However, within 15 min, a few cells showed vacuolation (Fig. 2A). After 30 min, the number of vacuolated fibers had increased and several hypercontracted cells were observed (Fig. 2B). Veratrine-treated muscle also showed interstitial edema which persisted for up to 60 min (Fig. 2B-C).

The NADH-TR reaction revealed alterations in the oxidative enzyme activity in all muscle fibers. In the subsarcolemmal and intermyofibrillar regions, the formazan deposits showed no corresponding increase or decrease in enzyme expression (Table 1). There was an increase in formazan aggregates along the subsarcolemmal region after 30 min, followed by a decrease to below control levels by 60 min (Fig. 2D-F). Conversely, the formazan deposits decreased until 30 min post-veratrine, then recovered slightly, although control

levels were not reached (Fig. 2D-F). The NADH-TR activity of red (oxidative) fibers decreased after veratrine. The reactivity of the oxidative-glycolytic fibers in the intermyofibrillar region increased slightly at 30 min and was sustained up to 60 min, whereas in type IIB fibers, the increase up to 30 min was followed by a slight decrease at 60 min (Fig. 2D-F). Anomalous accumulations of formazan particles were seen in a significant number of cells in the three fiber types (Fig. 2E). The changes in the intensity of the reaction are summarized in Table I and Fig. 3.

The levels of m-ATPase activity in the three fiber types 15 min and 30 min post-veratrine were similar to the controls (Fig. 3A-D). However, at 60 min, an unexpected fourth type of fiber appeared which showed no reversal of staining at both pH values (Figures 3E-F). Prominent interstitial edema occurred in the muscle tissue after contact with the alkaloid for 60 min.

Ultrastructural findings

Saline-treated fish: - Longitudinal sections of the lateral muscle of fish injected with sterile saline showed well-defined, aligned sarcomeres 1.45 μm in length. The A and I bands were arranged in register, and were bisected by the M and Z lines. The M-line in fish is thick and almost the same width as the H-band. The myofibrils (0.69 μm thick) were well delimited by a relatively wide intermyofibrillar space occupied by membranous profiles of the sarcotubular system, ribosomes and mitochondria in slow red fibers (type I). The triads were arranged at the Z-lines, which showed a zig-zag pattern (Fig. 4A). The T tubule was interposed between the two lateral, well-developed sarcoplasmic reticulum terminal cisternae (SR-TC) (Fig. 4A).

Veratrine-treated fish: - Veratrine produced a variety of alterations within 15 min which were maintained throughout the experiment (60 min). The changes were focal, with some fibers showing no sign of damage. Up to 30 min post-injection, the size of the affected areas was similar to that of unaffected areas, but then decreased by 60 min. Most changes were associated with the disorganization and size of the sarcomeres, which ranged from 1.38 μm to 1.61 μm in length and with the myofibrils. The thickness of the myofibrils (0.76 μm to 1.11 μm) also changed, the sarcomeres were disorganized and the

myofilaments appeared dissociated or ruptured (Figs. 4B, 4D and 5A). Degenerated mitochondria, which were swollen and irregular in shape, contained electronlucent flocculent material and disorganized cristae, especially after 15 min and 30 min (Fig. 4C). In some cells, mitochondria were more preserved (Figs. 4D, 4E and 5A). The T-tubules remained unaltered throughout the experiment (Figs. 4B, 4D and 5A). Although there was no remarkable change in the SR-TC, the network of the reticulum tubules showed discontinuities by 60 min (Fig. 5A). The M- and Z-lines assumed a tortuous, more accentuated osmyophilia in veratrinized muscles (Figs. 4B, 4D and 5A). In some cases, there was interruption of the M-lines (Fig. 5A) and an increased number of pinocytotic vesicles underlying the sarcolemma (Fig. 4B and 4E). Prior treatment with TTX prevented the alterations in myofibril and sarcomere organization, and decreased the number of pinocytotic vesicles (Fig. 5B). The T-tubules appeared intact, the SR-TC preserved its original dimensions and the network of SR tubules was again continuous and well developed (compare Fig. 5A and 5B).

DISCUSSION

As in other teleosts, the lateral musculature in juvenile tilapia (*O. niloticus*) consisted of a superficial layer of small-diameter fibers or "red" muscle overlying a deep layer of larger diameter, polygonal fibers known as "white" muscle. The red layer consisted of slow-contracting fibers with predominantly aerobic metabolism, and the deep layer, which accounted for most of the lateral muscle mass, consisted of fibers with mainly anaerobic pathways of metabolism. Red muscle is involved in routine activities such as foraging and migration, whereas white muscle is important during rapid acceleration and high-speed swimming (Kilarski, 1990; Johnston, 1994).

The intermediate layer of "pink" muscle located between the red and white muscles contains large and small diameter fibers (Mascarello et al., 1986). These fibers have oxidative, glycolytic and oxidative-glycolytic activities as shown by the NADH-TR reaction (Ogata and Mori, 1964; Patterson et al., 1975; Mascarello et al., 1986; Kilarski, 1990), are stable to m-ATPase activity after acidic or alkaline preincubations and have distinct forms of myosin based on immunohistochemistry (Mascarello et al., 1986). The great variation in staining intensities is correlated with the presence and varying expression of myosin isoforms during development (Rowlerson et al., 1985; Romanello et al., 1987; Mascarello et al., 1986; Staron, 1991). The variation in fiber diameter, which produced the mosaic appearance seen in tilapia, agrees with the hyperplastic growth of muscle that occurs in fish (Carpené and Veggetti, 1981; Egginton and Johnston, 1982; Rowlerson et al., 1985).

Veratrine increased the NADH-TR activity in white fibers, indicating greater oxidative activity, as confirmed by the formazan precipitate associated with mitochondria. In contrast, veratrine had an opposite effect on red oxidative fibers, i.e., the alkaloid depressed the oxidative enzyme reaction. Maximal change occurred at 30 min, with subsequent partially reversal during the next 30 min (see Table 1). NADH-TR activity rose moderately in the subsarcolemmal region and decreased to half the control values in the intermyofibrillar region of red fibers 30 min after veratrine. This response was also observed in oxidative-glycolytic and glycolytic fibers. These divergent mitochondrial

responses suggested the existence of distinct cell compartments or factors which modulate the metabolism of these organelles.

In previous work on the effects of veratrine in the *soleus* and EDL of mice, veratrine decreased the reactivity of all three fiber types, although oxidative and oxidative-glycolytic fibers were more sensitive than glycolytic fibers, with no difference between the *soleus* and EDL (Freitas et al., 2000b). Veratrine significantly increased the number of oxidative fibers in EDL and decreased the number of glycolytic fibers. In *soleus*, veratrine decreased the number of oxidative and oxidative-glycolytic fibers.

There was no change in the pattern of the m-ATPase reaction in any of the three fiber types in the three zones of the lateral muscle of juvenile tilapia up to 30 min after veratrine injection. The m-ATPase activity correlated with the occurrence of at least two myosin isoforms (acidic-stable in red muscle and alkaline-stable in the white muscle) up to 30 min post-veratrine. However, by 60 min, a marked change had occurred and another fiber type with no reversal of staining had appeared. Cells with the above characteristics have been classified as “undifferentiated cells” and have been reported in fish during development and after fluctuations in water temperature. In both cases, different isoforms of myosin are eventually expressed. Similar changes have been demonstrated in myofibrillar regulatory proteins of goldfish (Johnston, 1979), in carp light chain myosin (Crockford and Johnston, 1990), and in the expression (Gerlach et al., 1990) and structure (Hwang et al. 1990, 1991) of carp heavy chain myosin. In tilapia, a fourth type of fiber appeared after a very short exposure to veratrine (60 min).

Comparison of the histochemical findings following veratrine injection in tilapia and mice (Freitas et al., 2000b) indicated different metabolic and contractile responses of the fibers, which may be species-, muscle- and fiber-specific. The ultrastructural changes seen in these two species generally supported these divergent responses.

In tilapia, there was none of the characteristic swelling of the SR-TC seen in mice treated with myotoxins that affect sodium currents by altering Na^+ channel gating (see Mebs and Ownby; 1990, Mattiello-Sverzut and Cruz-Höfling, 2000) or with veratrine *in vitro* (Cruz-Höfling and Vital Brazil, 1990) and *in vivo* (Freitas et al., 2000b). The normal, wider SR-TC present in fish skeletal muscle (Franzini-Armstrong and Porter, 1964, for

review see Ogata, 1988) remained intact after veratrine or TTX+veratrine. However, veratrine produced discontinuities in the longitudinal compartment of the SR, a disturbance prevented by pretreatment with TTX. This part of the reticulum is involved in the active uptake of Ca^{2+} from the sarcoplasm through Ca^{2+} -ATPase activity during muscle relaxation. The stimulation of Ca^{2+} -ATPase and an increase in SR Ca^{2+} loading has been reported for myotoxin *a*, a Na^+ channel-acting peptide from the venom of the rattlesnake *Crotalus viridis viridis*, in isolated rabbit skeletal muscle SR (Volpe et al., 1986). The toxin bound to the SR membranes and uncoupled Ca^{2+} uptake from Ca^{2+} -dependent ATP hydrolysis. Myotoxin *a* caused vacuolation of muscle cells (Tu and Morita, 1983) through SR-TC swelling (Ownby et al., 1976), an effect not seen in tilapia lateral muscle cells.

The most prominent ultrastructural change was the damage to sarcomere and myofibrillar structure. This damage, which did not involve all muscle fibers, decreased by 60 min and was prevented by prior injection of TTX. Curiously, veratrine caused a time-dependent increase in the length of lateral muscle sarcomeres. In mouse (Vital Brazil and Fontana, 1985) and frog (Lehoueller et al., 1988) muscles, veratrine causes repetitive nerve firing, which leads to iterative muscle fiber depolarization and hypercontraction, as well as sarcomere shortening. Although light microscopy of tilapia muscle indicated hypercontracted zones, no hypercontracted sarcomeres were seen with TEM. The mitochondrial effects of veratrine in tilapia were similar to those in mouse EDL and *soleus*, with the damage showing partial reversal after 60 min. Veratrine can uncouple oxidative phosphorylation in mouse and rat isolated mitochondria (Freitas et al., 2000d). This effect may reflect a nonspecific mitochondrial permeability transition induced by veratrine, perhaps through mitochondrial Ca^{2+} overload, a suggestion supported by the state of relaxation indicated by the sarcomere lengthening.

There are few reports of the morphological and physiological responses of fish to toxic environmental conditions resulting from river pollution or chemical dumping. Most investigations have dealt with the biochemical and physiological responses of skeletal muscle in fish during developmental stages or in response to changes in temperature. There are no reports on the effects of veratrine in fish skeletal muscle. The results of this study indicate that the mechanism of action of the veratrine in tilapia is distinct from that in mice,

although some of the disturbances in muscle enzyme activity and morphological alterations are superficially similar.

Acknowledgements: The authors thank Mr. Gustavo H. Silva, Mrs. Sueli Michelin and Mrs. Maria Helena Moreno for valuable technical assistance. This work was supported by grants from CAPES, CNPq, FAPESP and FAEP-UNICAMP, Brazil.

REFERENCES

- BENFORADO, J. M. The veratrum alkaloids. In: *Physiological pharmacology*. (Root, W. S. and Hofmann, F. G. Eds.). New York. Academic Press, pp. 331-398, 1967.
- BROOKE, M. H. and KAISER, K. K. Muscle fiber types: how many and what kind? *Arch. Neurol.* **23**: 369-379, 1970.
- CARPENÈ, E. and VEGGETTI, A. Increase in muscle fibers in the *lateralis* muscle (white portion) of Mugilidae (Pisces, Teleostei). *Experientia* **37**:191-193, 1981.
- CROCKFORD, T. and JOHNSTON, I. A. Temperature acclimation and the expression of contractile protein isoforms in the skeletal muscles of the common carp (*Cyprinus carpio* L.). *J. Comp. Physiol. B* **160**: 23-30, 1990.
- CRUZ-HÖFLING, M. A. and VITAL BRAZIL, O. Alterações ultraestruturais causadas pela veratrina no diafragma isolado de camundongo. [Abstract] In: *Proceedings of the V Annual Meeting of FESBE*, p 324, Caxambu, MG, Brasil, 1990.
- EGGINTON, S. and JOHNSTON, I. A. A morphometric analysis of regional differences in myotomal muscle ultrastructure in the juvenile eel. *Cell Tiss. Res.* **222**: 579-596, 1982.
- FRANZINI-ARMSTRONG, C. and PORTER, K. R. Sarcolemmal invaginations constituting the T system in fish muscle fibers. *J. Cell Biol.* **22**: 675-696, 1964.
- FREITAS, E. M. S., SILVA DAL PAI, M. and CRUZ-HÖFLING, M. A. A histochemical study of the interaction of mouse *extensor digitorum longus* and *soleus* muscles with veratrine. 2000a. *Exp. Neurol.* (submitted).
- FREITAS, E. M. S., SILVA DAL PAI, M. and CRUZ-HÖFLING, M. A. Mouse *extensor digitorum longus* and *soleus* show distinctive ultrastructural changes induced by veratrine. 2000b. *Exp. Neurol.* (submitted).
- FREITAS, E. M. S., SILVA DAL PAI, M. and CRUZ-HÖFLING, M. A. Effects of veratrine on respiratory activity of isolated muscle and liver mitochondria. 2000c. (submitted).

- GERLACH, G., TURAY, L., MALIK, K. T. A., LIDA, J., SCUTT, A. and GOLDSPINK, G. Mechanisms of temperature acclimation in the carp: a molecular biology approach. *Am. J. Physiol.* **259**: R237-R244, 1990.
- GUTH, L. and SAMAHA, F. Qualitative differences between actomyosin ATPase of slow and fast mammalian muscle. *Exp. Neurol.* **25**: 138-152, 1969.
- HWANG, G. C., WATANABE, S. and HASHIMOTO, K. Changes in carp myosin ATPase induced by temperature acclimation. *J. Comp. Physiol. B* **160**: 233-239, 1990.
- HWANG, G. C., OCHIAI, Y., WATANABE, S. and HASHIMOTO, K. Changes of carp myosin subfragment-1 induced by temperature acclimation. *J. Comp. Physiol. B* **161**: 141-146, 1991.
- JOHNSTON, I. A. Calcium regulatory proteins and temperature acclimation of actomyosin ATPase from a eurythermal teleost (*Carassius auratus* L.). *J. Comp. Physiol. B* **129**: 163-167, 1979.
- JOHNSTON, I. A. Development and plasticity of fish muscle with growth. *Basic Appl. Myol.* **4**: 353-368, 1994.
- KILARSKI, W. Histochemical characterization of myotomal muscle in the roach, *Rutilus rutilus* (L.). *J. Fish Biol.* **36**: 353-362, 1990.
- LEHOUELLER, J., LASZZYCA, P. and BRUNES, J. Sodium-dependent depolarizing potentials in veratrinized crayfish muscle fibres. *Neurosci. Lett.* **89**: 299-304, 1988.
- MASCARELLO, F., ROMANELLO, M. G., SCAPOLO, P. A. Histochemical and immunohistochemical profile of pink muscle fibers in some teleosts. *Histochemistry* **84**: 251-255, 1986.
- MATTIELO-SVERZUT, A. C. and CRUZ-HÖFLING, M. A. Toxin 2 (PhTx 2), a neurotoxic fraction from *Phoneutria nigriventer* spider venom, causes acute morphological changes in mouse skeletal muscle. *Toxicon* **38**: 793-812, 2000.
- MEBS, D. and OWNBY, C. L. Myotoxic components of snake venoms: their biochemical and biological activities. *Pharmacol. Ther.* **48**: 223-236, 1990.

- NÁNÁSI, P. P., VARRO, A., LATHROP, D. A. and BRYONT, S. H. Effects of veratridine on Na^+ and Ca^{2+} currents in frog skeletal muscle. *Gen. Pharmacol.* **25**: 1661-1666, 1994.
- OGATA, T. Morphological and cytochemical features of fiber types in vertebrate skeletal muscle. *CRC Crit. Rev. Anat. Cell Biol.* **1**: 229-275, 1988.
- OGATA, T. and MORI, M. Histochemical study of oxidative enzymes in vertebrate muscles. *J. Histochem. Cytochem.* **12**: 171-182, 1964.
- OWNBY, C. L., CAMERON, D. and TU, A. T. Isolation of a myotoxic component from rattlesnake (*Crotalus viridis viridis*) venom. *Am. J. Pathol.* **85**: 149-166, 1976.
- PATTERSON, S., JOHNSTON, I. A. and GOLDSPINK, G. A histochemical study of the lateral muscle of five teleost species. *J. Fish Biol.* **7**: 159-166, 1975.
- PETER, J. B., BARNARD, R. J., EDGERTON, U. R. and GILLESPIE, C. A. and STEMPLE, K. E. Metabolic profiles of three types of fibers of skeletal muscles in guinea pig and rabbits. *Biochemistry* **11**: 2627-2633, 1972.
- ROMANELLO, M. G., SCAPOLO, P. A., LUPRANO, S. and MASCARELLO, F. Post-larval growth in the lateral white muscle of the eel *Anguilla anguilla*. *J. Fish Biol.* **30**: 161-172, 1987.
- ROWLERSON, A., SCAPOLO, P. A., MASCARELLO, F., CARPENÈ, E. and VEGGETTI, A. Comparative study of myosin present in the lateral muscle of some fish species: variations in myosin isoforms and their distribution in the pink and white muscle. *J. Muscle Res. Cell Motil.* **6**: 601-640, 1985.
- STARON, R.S. Correlation between myofibrillar ATPase activity and myosin heavy chain composition in single human muscle fibres. *Histochemistry* **96**: 21-24, 1991.
- SUTRO, J. B. Kinetics of veratridine action on Na^+ channels of skeletal muscle. *J. Gen. Physiol.* **87**: 1-24, 1986.
- TU, A. T. and MORITA, M. Attachment of rattlesnake venom myotoxin *a* to sarcoplasmic reticulum: peroxidase conjugated method. *Br. J. Exp. Pathol.* **64**: 633-637, 1983.

-
- ULBRICHT, W. The effect of veratridine on excitable membranes of nerve and muscle. *Ergeb. Physiol. Biol. Exp. Pharmacol.* 61: 18-71, 1969.
- ULBRICHT, W. Effects of veratridine on sodium currents and fluxes. *Rev. Physiol. Biochem. Pharmacol.* 133: 1-54, 1998.
- VITAL BRAZIL, O. and FONTANA, M. D. Unequal depolarization of the membrane of the rat diaphragm muscle fibers caused by veratrine. *Pflügers Arch.* 404: 45-49, 1985.
- VOLPE, P., DAMIANI, E., MAURER, A. and TU, A. T. Interaction of myotoxin a with the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum. *Archs. Biochem. Biophys.* 246: 90-97, 1986.

LEGEND OF FIGURES

Figure 1: *Cross-sections of the lateral muscle of *O. niloticus*.*

(A) Control:- Red muscle (R) and white muscle (W). HE; (B) oxidative (ox), glycolytic (gly) and oxidative glycolytic (og) fibers as identified by the NADH-TR reaction; (C-D) m-ATPase reaction at pH 4.6 and 10.2, respectively:- type I, IIA and IIB muscle fibers, organized as a mosaic, are present in the intermediate layer between red and white muscles. Bar = 50 μ m for all panels.

Figure 2: *Light micrographs of the lateral muscle of *O. niloticus*.*

Fifteen minutes (A and D), 30 min (B and E) and 60 min (C and F) after the injection of veratrine (5 μ l, 10 ng/kg fish). (A-C) H&E. Vacuolated cells (v), hypercontracted fibers (h), interstitial edema (asterisks); (D-F) NADH-TR reaction showing muscle fibers with subsarcolemmal or central aggregates of formazan particles (arrowheads). Bar = 50 μ m for all panels.

Figure 3: *Light micrographs of m-ATPase of the lateral muscle of *O. niloticus*.*

Fifteen minutes (A-B), 30 min (C-D) and 60 min (E-F) after veratrine injection. Muscle fiber types I, IIA and IIB reacted after preincubation at pH 4.6 (A, C and E) and pH 10.2 (B, D and F). Note the presence of undifferentiated cells (*), interstitial edema and muscle tissue disarray. Bar = 50 μ m.

Figure 4: *Electronmicrographs of the lateral muscle of *O. niloticus*.*

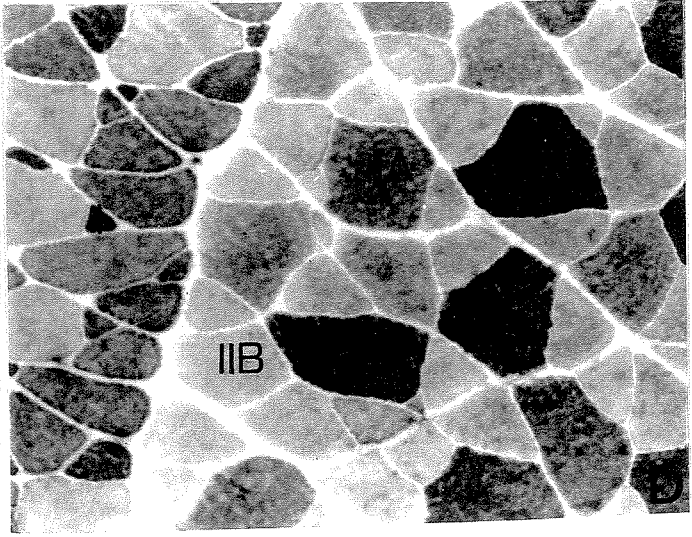
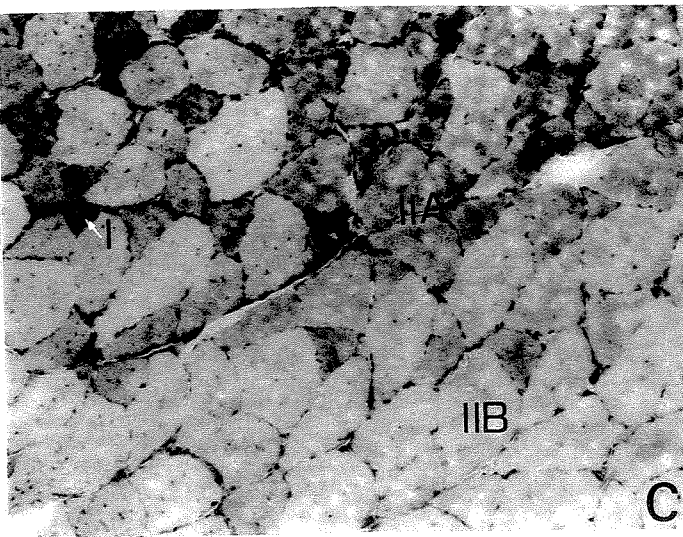
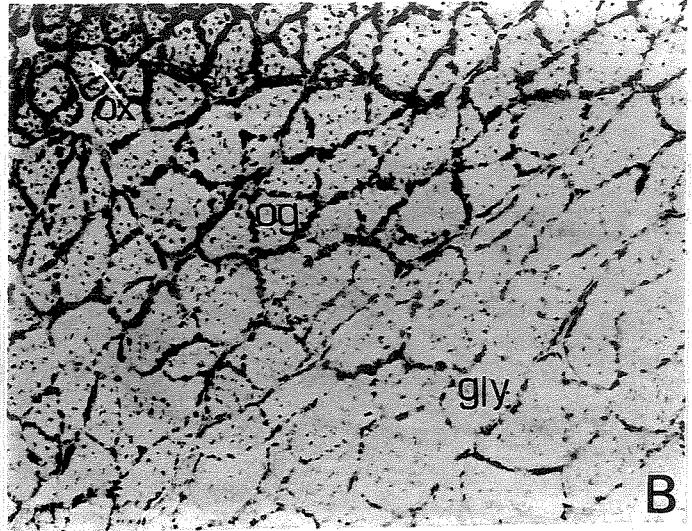
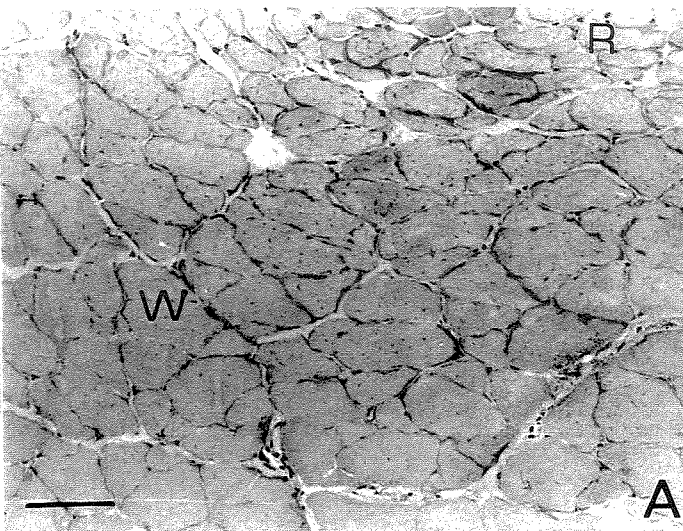
(A) Control muscle. Sarcomeres (s), myofibrils (m), Z-line (Z) showing the zig-zag pattern (arrow), dense and large M-line (M), triads (tr), muscle fibers 15 min (B-C) or 30 min (D-E) after veratrine (5 μ l, 10 ng/kg) injection. Note the dissociation of the myofilaments showing the underlying longitudinal network of SR tubules (*), sarcoplasmic reticulum

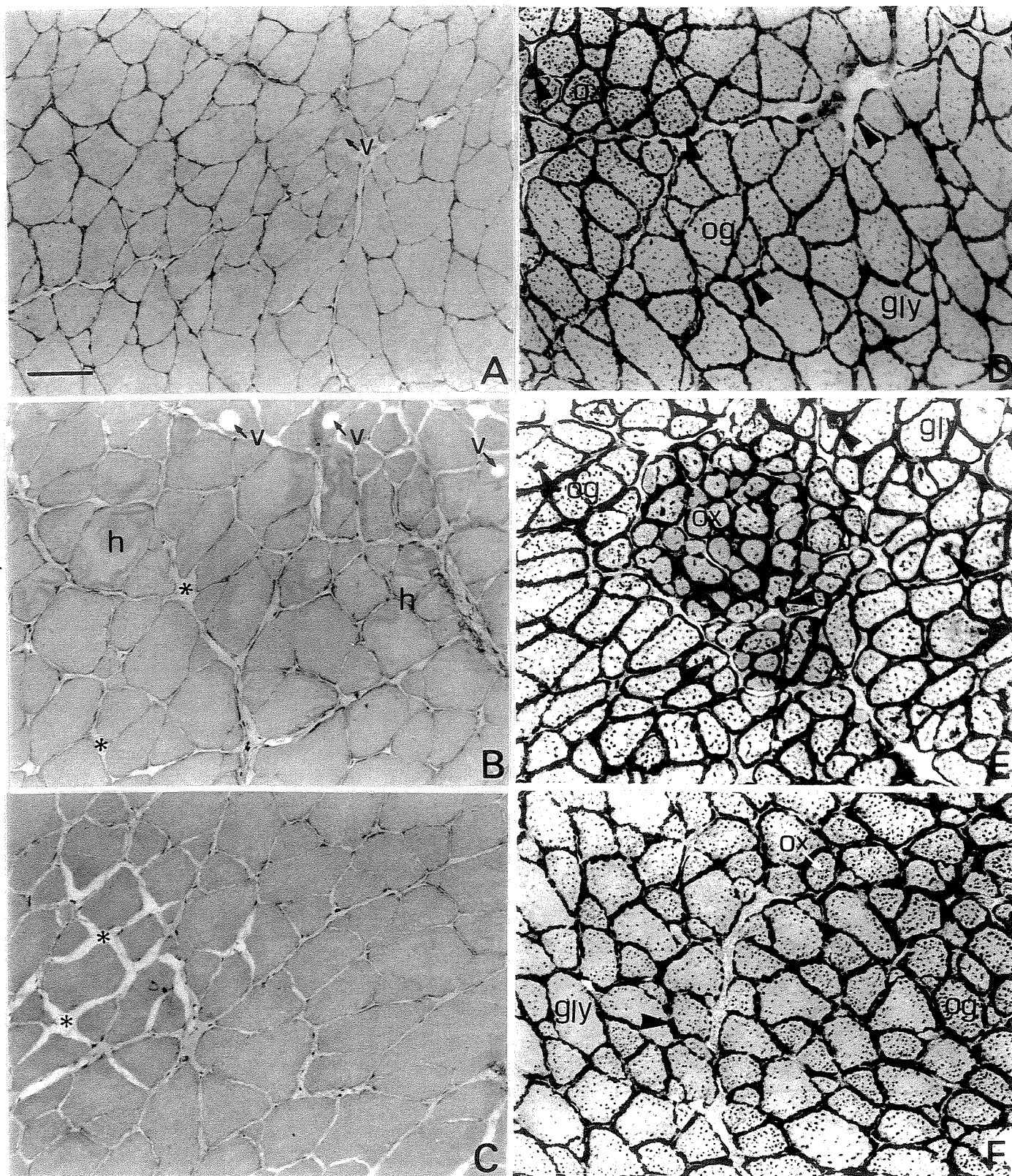
terminal cisternae (sr), T-system (t), and abnormal mitochondria (mi). Bars = 0.5, 0.5, 0.25, 0.5 μm , respectively.

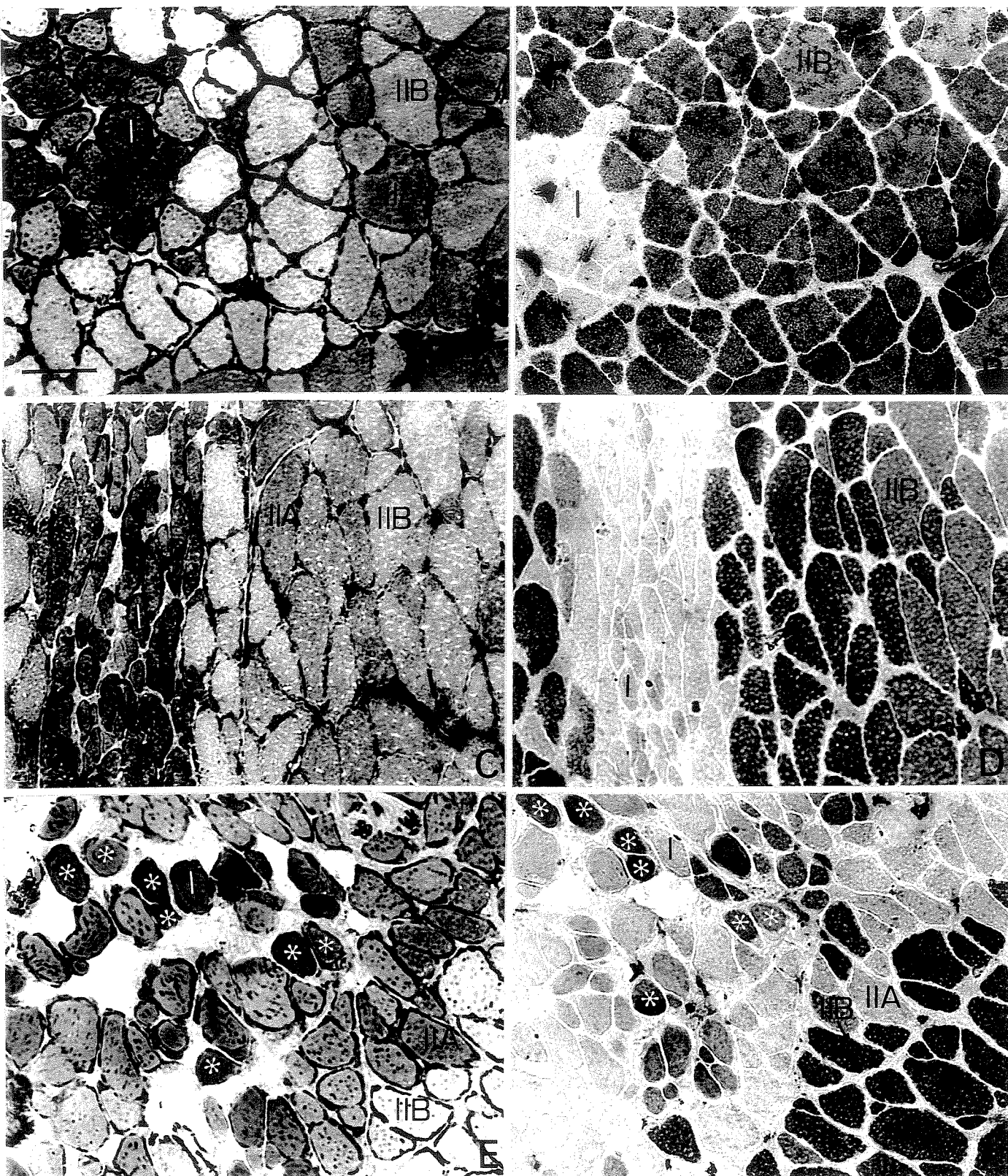
Figure 5: *Electronmicrographs of the lateral muscle of O. niloticus.*

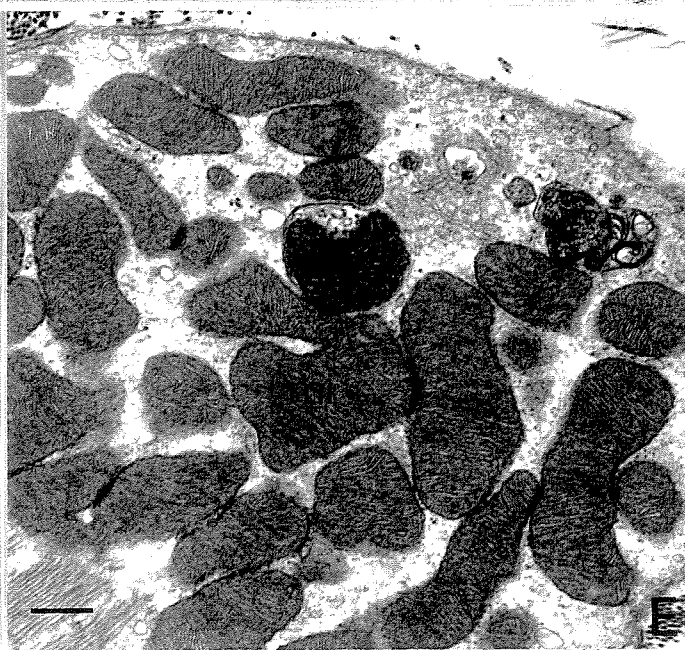
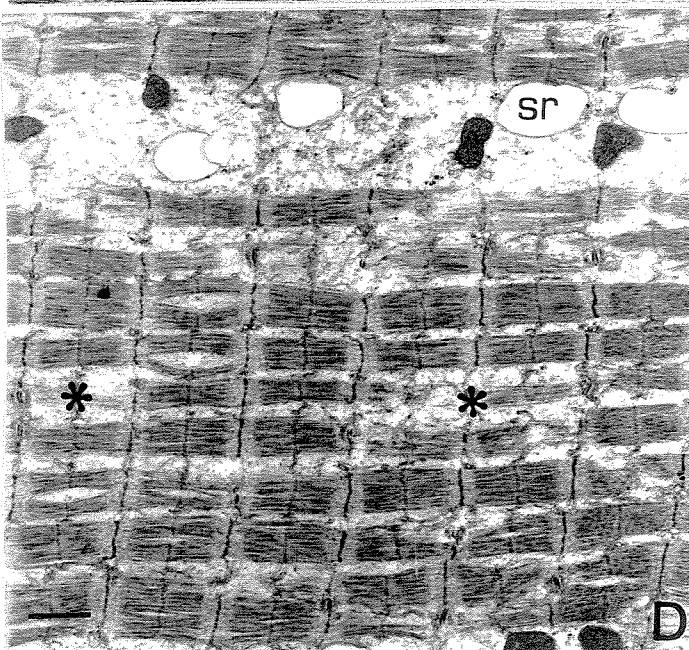
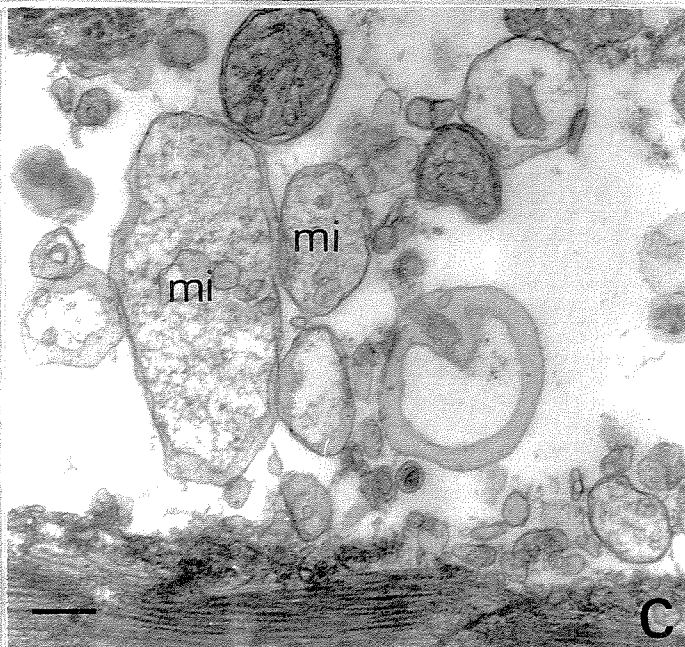
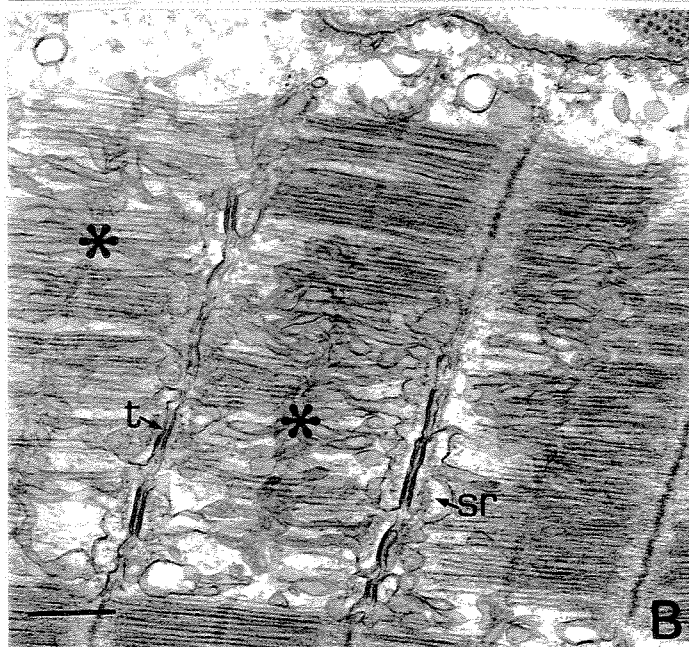
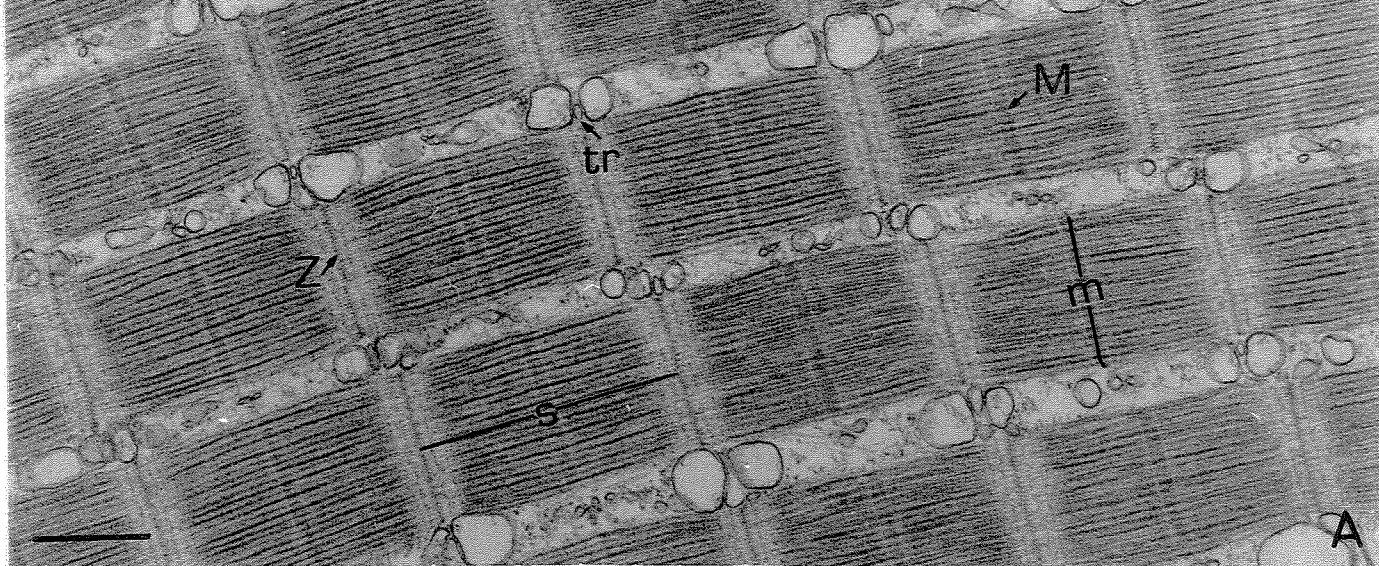
(A) 60 min after veratrine injection; (B) TTX-treated (1 ng/ml) fibers before veratrine injection (60 min survival time). Note the dissociated or ruptured myofilaments (*), sarcoplasmic reticulum cisternae (sr), T-system (t), mitochondria (mi), collagen fibers (fc), and distorted Z- lines (Z). Bars = 1.0 μm and 0.125 μm , respectively.

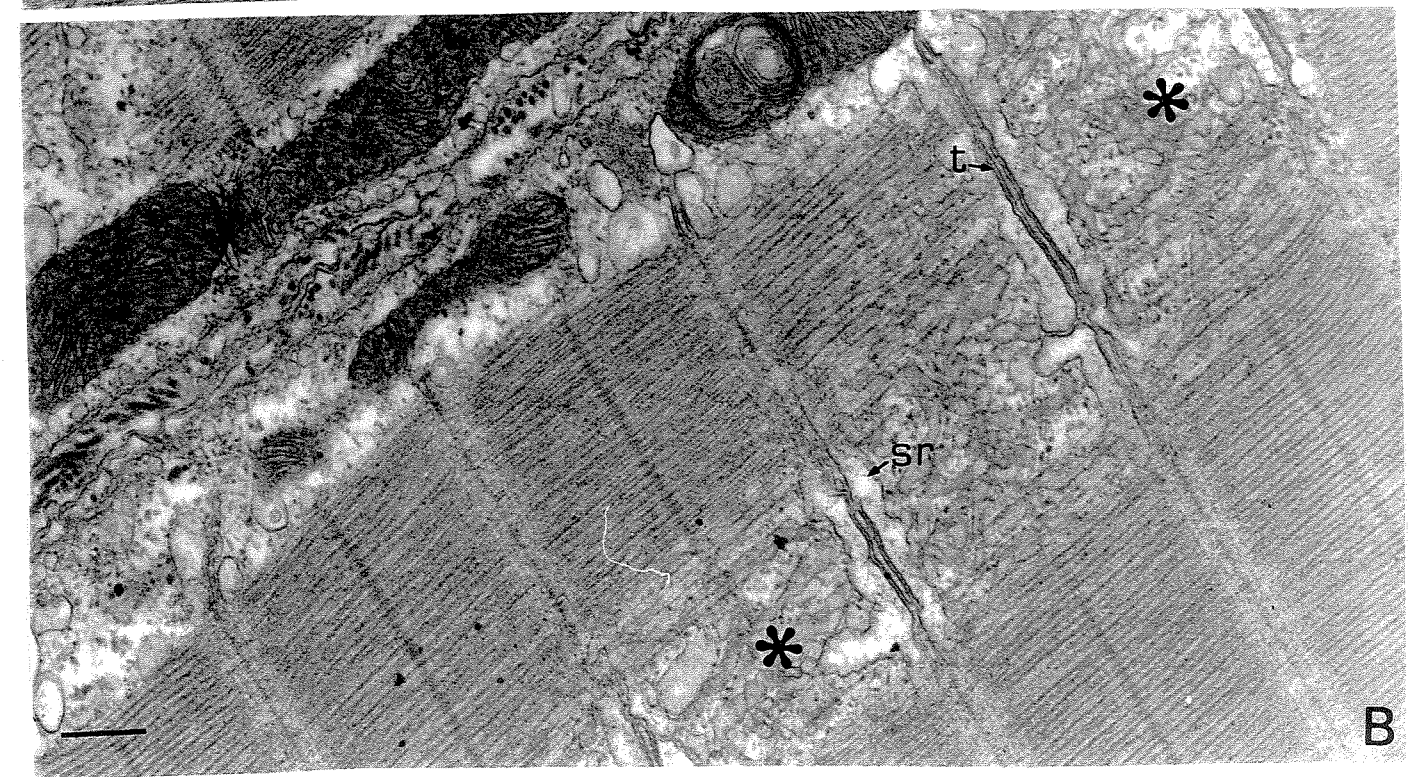
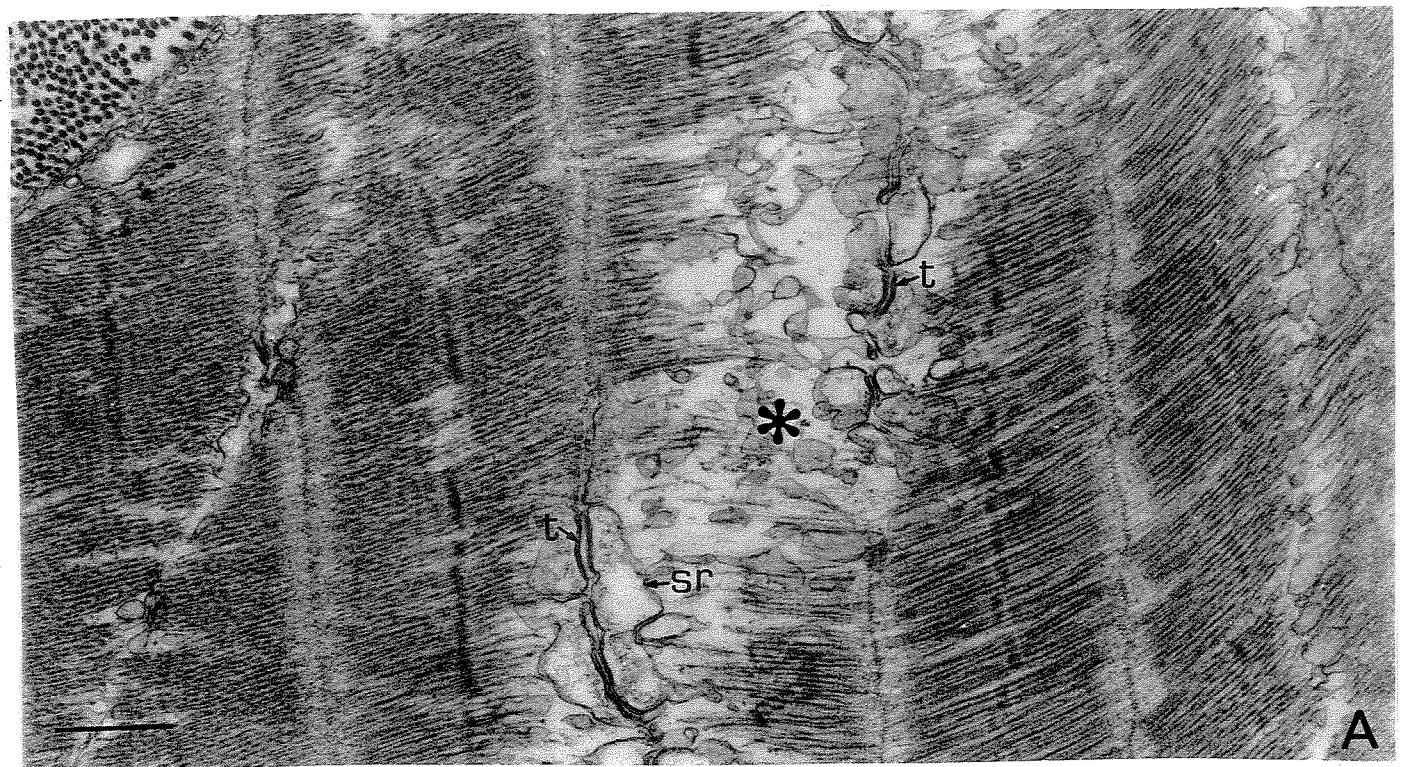
Table 1: Formazan particle distribution in the subsarcolemmal and intermyofibrillar region of muscle cells in type I, IIA and IIB fibers 15, 30 and 60 min after the injection of veratrine.











FIBER TYPE	TREATMENT WITH VERATRINE X TIME				
		CONTROL	15 MIN	30 MIN	60 MIN
I	S	+++	+++	++++	++
	I	++++	+++	++	+++
IIA	S	++	++	+++	+++
	I	++	++	+++	++++
IIB	S	+	++	++	++
	I	+	+	+++	++

S= Subsarcolemmal ; I=Intermyofibrillar

✉ Maria Alice da Cruz-Höfling
Departamento de Histologia e Embriologia
Instituto de Biologia – C.P. 6109
Universidade Estadual de Campinas
13 087-130 Campinas – SP – BRASIL
email: hofling@obelix.unicamp.br

EFFECTS OF VERATRINE ON THE RESPIRATORY ACTIVITY OF ISOLATED MUSCLE AND LIVER MITOCHONDRIA

¹Erika Maria Silva Freitas, ²Márcia M. Fagian, ²Anibal Eugênio Vercesi,

✉¹Maria Alice da Cruz Höfling

¹Departamento de Histologia e Embriologia, Instituto de Biologia, and Departamento de
Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas
(UNICAMP), Campinas, SP, BRASIL

Running Title: Mitochondrial effects of the veratrine alkaloid

ABSTRACT

In this paper, we have focused our attention on the effects of veratrine, a steroidal substance of alkaloid nature, in skeletal muscle mitochondria of mice *in vivo*, and in isolated mitochondria of mice and rats *in vitro*. Veratrine caused various degrees of degeneration in mitochondria from mouse *extensor digitorum longus* (EDL) and *soleus* (SOL) muscles when injected *in situ*. Ultrastructural alterations were time-dependent and at certain extent also muscle-specific. They were short-lived being almost resolved by 60 min post-veratrine. These morphological changes were attributed to a higher influx of Na^+ and water into muscle cells induced by veratrine (a Na^+ channel-activating toxin), and supposedly leading to greater mobilization of Ca^{2+} ions from mitochondria. *In vitro*, veratrine provoked the uncoupling of the mice and rats skeletal muscle mitochondria respiration and the inhibition of the rats liver mitochondria respiration. The preliminary biochemical assays showed that veratrine acts on the permeability transition of mitochondria inner membrane unspecifically. A role of the veratrine in the mobilization of calcium stores from mitochondria is raised.

Key Words: Mitochondria, skeletal muscle, liver, uncoupling, respiratory inhibition, ultrastructural changes, veratrine.

INTRODUCTION

Recent evidences indicate that the alkaloid veratrine may be involved in degenerative mitochondrial changes observed when the drug is injected intramuscularly in mice (Freitas et al., 2000 a). Veratrine, extracted from the seeds of some plants of the family Liliaceae (Ulbricht, 1998), is a mixture of mainly two alkaloid esters, veratridine and cevadine (Benforado, 1967). It activates Na^+ channels of excitable membranes (Catterall, 1980) causing iterative depolarization (Vital Brazil and Fontana, 1985), higher influx of Na^+ and hence an osmotic accumulation of fluid inside the cell which, morphologically, appears as numerous tiny vacuoles inside muscle cells or as peri- or intra-axonal or intra-myelin vacuoles in nerve fibers (Cruz-Höfling and Vital Brazil, 1990). In view of evidences indicating a probable access of the toxin into the cell (Honerjäger et al., 1992), facilitated by its lipid-soluble biochemical property (Catterall, 1980) and corroborated by suggestive differences on the action of veratrine in *soleus* (predominantly composed of oxidative muscle fibers) and in *extensor digitorum longus* (EDL, predominantly composed of glycolytic muscle fibers) (Freitas et al., 2000a) we have extended our observations on the effects of veratrine in isolated mitochondria. In this work, we will correlate the findings on the effects of veratrine in the respiratory activity of isolated muscle mitochondria from the hind limb skeletal muscles from rats and mice to those obtained from rat liver mitochondrial activity assays screened for comparative studies. An improved understanding of the targets of veratrine interaction and the mechanisms behind the morphofunctional disturbances of muscle tissue should facilitate the comprehension of distinctive differences of *soleus* and EDL responses seen elsewhere (Freitas et al., 2000a).

The present study has undertaken to investigate oxygen consumption on isolated mitochondria from hind limb muscles and liver after addition of the Na^+ channel-activating alkaloid veratrine. The biochemical assay was combined with ultrastructural studies of EDL and *soleus* mitochondria on saline- and veratrine-injected mice.

MATERIALS AND METHODS

Animals

Adult BALB/c mice (20-25 g) and Wistar strain rats (200-250 g) from the Animal House Unit of the State University of Campinas (UNICAMP, Campinas, SP, Brazil) were used.

Toxin

Veratrine was commercially obtained from Sigma Chemical Co., St. Louis, MO, USA. The toxin was used in the dose 10 µg/ml on experiments *in vivo*, and in the dose of 10 to 50 µg/ml on biochemical assays.

Ultrastructural studies

Anesthetized mice with sodium pentobarbital (40-70 mg/kg) were submitted to a microsurgery, which expose the *extensor digitorum longus* (EDL) and *soleus* (SOL) muscles for receiving an *in situ* injection of 5 µl veratrine (10 ng veratrine/kg mice). After 15, 30 or 60 min (n=2/groups), mice under deep anesthesia were perfused through the left ventricle with Karnovsky fixative (2.5 % glutaraldehyde plus 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The muscles were removed and sectioned into small fragments. Muscle fragments were immersed in Karnovsky solution, post-fixed in 1% OsO₄, dehydrated through graded acetone and processed for transmission electron microscopic (TEM) examination. Control group received sterile saline solution injection at the same experimental condition.

Isolation of mouse and rat skeletal muscle mitochondria

Male mice and rats were sacrificed through cervical dislocation before hind limbs muscles were removed. The muscles were dissected, perforated and immersed in ice-cold isolation buffer with the following composition: 0.15 M KCl, 10mM Hepes (n-2-hydroxyethylpiperazine-N'-2 ethanosulfonic acid), 1.0 m M EGTA (ethylene-glycol-bis-(β-aminoethylene)-N-N-N'-N'-tetracetic) and 0.5% BSA (bovine serum albumin), pH 7.2 at 4°C. After, the muscles were washed in ice-cold medium, finely minced and homogenized

(Polytron, 4 second at setting 3; Van Potter, 3 strokes) in the same buffer. The homogenate was centrifuged (4°C) at 800xg for 8 min (twice). The supernatant was retrieved and centrifuged at 7000xg for 10 min. The resulting pellet was then resuspended in the same buffer lacking EGTA and recentrifuged at 7000xg for 10 min.

The final pellet was resuspended in the EGTA-free buffer to a protein concentration of 30-50 mg/ml and kept on ice during all biochemical assays. Protein concentration were determined by the biuret method using BSA as standard.

Isolation of rat liver mitochondria

Rat liver mitochondria (RLM) were isolated by conventional differential centrifugation from the livers of adult female Wistar rats fasted overnight, using a buffer containing 1.0 M sucrose, 0.5 M Hepes and 0.1 M EGTA. The mitochondria were washed and resuspended in the same buffer without EGTA. Protein concentration (80-100 mg/ml) was determined by the biuret method.

Biochemical Assays

Oxygen consumption was measured with a Clark-type electrode (Yellow Springs Instruments Co., OH, USA) in 1.3 ml of standard incubation medium (28°C), in a sealed glass cuvette equipped with a magnetic stirrer. The incubation medium contained 125 mM sucrose, 65 mM KCl, 10 mM Hepes, pH 7.2, 0.5 mM EGTA, 2 mM Pi and 1.0 mM MgCl₂, with addition to veratrine and 0.3 and 1.0 mg of muscle or liver mitochondrial protein, respectively. Each experiment was performed in triplicate.

RESULTS

Ultrastructural findings

EDL

Control: In the EDL muscle the mitochondria were normal in appearance, keeping cristae and matrix with normal morphology. They were aligned along the myofibrils, laterally to the Z-lines, at the I bands and having side-by-side the triads. The cristae were regular and the matrix electrondense (Fig. 1A).

Veratrinized EDL: Veratrine induced remarkable changes in the EDL mitochondria. The pathogenesis of the changes started at 15 min with an initial moderate increase of the organelle dimension which persisted time-dependently up to 60 min. The changes involved all the components, matrix and cristae as described in detail in the legend for figures (Fig. 1B-F). Ruptured mitochondria were seen by 30 min. A regression of the changes had its onset after 60 min of veratrine treatment (Fig. 1F).

SOL

Control soleus: As for EDL, the mitochondria were structurally normal, being found in the intermyofibrillar (Fig. 2A) and subsarcolemmal space.

Veratrinized soleus: Soon at 15 min, SOL mitochondria showed high degree of degeneration characterized by increase of volume, rupture and partial loss of cristae (Fig. 2A). In the *soleus* the disruption of the mitochondria is most common than in the EDL (Fig. 2B-2E). A detailed description of the abnormalities is found in the legend for figures. As for EDL these alterations were short-lived, as they were greatly resumed by 60 min, although some swelling persisted, particularly in the mitochondria population of the subsarcolemmal regions (Fig. 2F). No indication of calcium accumulation was seen inside the mitochondria of EDL and *soleus*.

Respiration rate in mice and rats muscle mitochondria

The state 4 respiration rate (20 nanoatoms oxygen/min/mg of protein for mice, and 44 for rats) and state 3 respiration rate (131 nanoatoms oxygen/min/mg of protein for mice, 253 for rats) (Figures 3 and 4 - trace A) confirmed the good coupling of the mice and rats

mitochondria (respiratory control factor - RC = 6.5 and 5.75, respectively). After addition of veratrine (10 $\mu\text{g/ml}$) in the incubation medium with isolated mice mitochondria, the state 3 respiration rate decreased (101 nanoatoms of oxygen/min/mg of protein) and the state 4 increased (55), leading to a reduction of the RC (1.8) (Figure 3 - trace B). In the assays with isolated rats mitochondria after contact with veratrine (50 $\mu\text{g/ml}$), there was an increase in the states 3 and 4 respiration rate (396 and 121, respectively) (Figure 4- trace B), showing a reduction of the RC (3.27), as seen in the mice.

Respiration Rate in rat liver mitochondria

Differently than it was seen in the mice and rats mitochondria, the state 3 respiration rate increased (from 108 to 110 nanoatoms oxygen/min/mg of protein) after addition of veratrine (50 $\mu\text{g/ml}$). The state 4 respiration rate decreased (25 from to 7.7 nanoatoms oxygen/min/mg of protein), leading to an increase of the RC (14.2).

DISCUSSION

The results indicate that there exist an interference of the veratrine in the RC of rats and mice muscle isolated mitochondria. However, whilst in mice the state 3 respiration rate decreased and the state 4 increased, in rats an increase in the state 3 and 4 respiration rate has occurred, but the final result of both was a reduction of the RC. In the liver though, the state 3 respiration rate increased after addition of veratrine (50 $\mu\text{g/ml}$), and the state 4 respiration rate decreased leading to an increase of the RC. Morphological correlation undertaken to investigate *in vivo* whether veratrine is able to disturb the structural organization of mitochondria, even when applied in the intercellular compartment of the muscle tissue of the EDL and soleo, confirmed the previous findings (Freitas et al., 2000 a,b).

Morphologically, veratrine induced various structural changes affecting not only the mitochondrial compartments, but also the outer and inner membrane of the mouse muscle mitochondria when injected *in situ*. Different patterns of mitochondrial alterations, as showed in our experiments, is usually described on reports of neuromuscular disorders (Lindal et al., 1992; Nishino et al., 1998), and on studies with use of uncouplers of oxidative phosphorylation (Melmed et al., 1975). Crotoxin, a neurotoxic fraction isolated from *Crotalus durissus terrificus* rattlesnake venom, and 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, caused mitochondria abnormalities, as swelling and disruption of cristae which appeared from 10-15 min injection up to 1 h (DNP), and 24 h (crotoxin) (Shah et al., 1982). The authors also observed intramitochondrial calcium after injection of these agents, suggesting that calcium ions accumulation may play a significant role in the pathogenesis of these alterations. Recent studies showed that veratrine causes necrosis in mice and fish muscle fibers characterized by hypercontraction of myofibrills, swelling of sarcoplasmic reticulum cisternae, disorganization and/or disruption of sarcomeres, mitochondrial damage, and that these ultrastructural alterations were prevented with tetrodotoxin pretreatment, a Na^+ channel-blocker (Freitas et al., 2000 a; c). These data seem to indicate that the majority of the alterations *in vivo* has its basis on a higher influx of Na^+ and water into cells, however the authors also suggested that a cell influx of Ca^{2+} ions, mobilized directly or indirectly by veratrine, could contribute to cell injury.

Biochemically, the mitochondrial myopathies can be divided into defects of substrate transport, utilization, oxidation-phosphorylation coupling, and respiratory chain (Lindal et al., 1992). Moreover, types of mitochondrial changes are variable from cell to cell and from individual to individual, resulting from gene expression of respiratory chain enzymes (Koga et al., 1988). Routine histoenzymological methods have shown that muscle biopsies specimens from patients with myopathies have high enzyme activities for NADH-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH) and cytochrome *c* oxidase (COX) in the subsarcolemmal region, and decreased in the sarcoplasm (Shah et al., 1982; Koga et al., 1988; Nishino et al., 1998).

Recently, we have shown that in the lateral musculature of teleost *Oreochromis niloticus*, veratrine affected differently the oxidative activity seen by NADH-TR reaction in the subsarcolemmal and intermyofibrillar regions and hypothesized on the existence of mitochondria populations whose internal stimuli should be under different control mechanisms or factors (Freitas et al., 2000b; c). On the other hand, contractile activity evaluation on *soleus* and EDL muscles from mice showed that oxidative and oxidative-glycolytic fibers were more susceptible to the action of the veratrine than glycolytic fibers, and that the muscle type (soleo: oxidative, slow twitch, and the EDL, glycolytic, fast twitch muscle) would regulate the response of a same fiber type, increasing their number in one muscle type, and decreasing it in the other (Freitas et al., 2000b). Similarly, the species/class of animal seemed to have a role in the response of muscle fiber to veratrine. So, in fish, oxidative and glycolytic fibers were strongly affected, differently to what occurs in mice (Freitas et al., 2000c). On the other hand, we confirmed that mitochondria of *soleus* muscle was early affected by the veratrine than EDL, which apparently were more resistant to the action of the alkaloid (Freitas et al., 2000a).

Biochemical assays with rats and mice skeletal muscle and rat liver mitochondria revealed changes on oxygen consumption after addition of veratrine. Veratrine caused uncoupling of oxidative phosphorylation, represented by increase of oxygen consumption during respiratory state 4 on mitochondria isolated from mice (starting from 10 µg/ml) and rats (50 µg/ml) muscles. On the contrary, veratrine (50 µg/ml) inhibited oxygen consumption on liver isolated mitochondria. As for the other different specificities aforementioned on the interaction veratrine vs classes of animals, type of muscle, type of

muscle fiber, here it was demonstrated that veratrine also interacts differently with mitochondria, depending on the organ, even though belonging to the same animal species (e.g. it exhibits opposite effects on the respiratory activity in the liver and muscle isolated mitochondria).

As mitochondrial membrane is devoid of Na⁺ channels, probably the veratrine, a lipid-soluble alkaloid toxin, interacts with their inner membrane, after transposing the outer membrane. The outer mitochondrial membrane is permeable to molecules of approximately 7 000 Da. It is constituted by phospholipids and the canal proteins porinas and several enzymes that interfere with lipid metabolism (e.g., lipases and sinthases). Its main function is to be responsible for a selective transport of energetic substrates, proteins and fatty acids. A presumable explanation for the effects in the physiology of the mitochondria here observed could be regarded as owing to the action of these alkaloids on cell membrane permeability. Pemberton et al. (1995) have demonstrated that the veratridine, a component of the veratrine, inhibits the refilling of cholinergic synaptic vesicles with acetylcholine in rats hemidiaphragm owing to its ability in dissipating the transvesicular membrane proton gradient in a non-specific way. Whether this proton quenching activity of veratridine (veratrine) can be correlated with the present observations on the ability of the alkaloid in accelerates the respiratory activity is until now uncertain. On the other hand, why in the liver this supposed quenching activity of the veratrine is not being expressed ?

Concluding the results indicate that veratrine uncouples oxidative phosphorylation in mitochondria of muscles. Whilst the precise mechanism by which veratrine inhibits the respiratory activity in the rat liver mitochondria, and enhances the respiratory activity in the rat and mouse muscle mitochondria remains to be elucidated, it appears, at least in part, that an action on the mitochondrial permeability transition to protons could be involved. Also, the studies pointing to the cell targets for veratrine interaction as the sarcoplasmic reticulum (Freitas et al., 2000 a) and the mitochondria (Freitas et al., 2000a, b, c) seem to suggest for an interference of the alkaloid in calcium metabolism.

Acknowledgments: The authors wish to express their cordial thanks to Mrs. Luciana P. Le Sueur for her helpful technical assistance, and to Dr. Stephen Hyslop for revising the

manuscript. This work was supported by grants from FAPESP, CNPq, CAPES and FAEP-UNICAMP.

REFERENCES

- BENFORADO, J. M. The veratrum alkaloids. In: ROOT, W. S. & HOFMANN, F. G. ed. *Physiological pharmacology*. New York, Academic Press. 1967. pp. 331-398.
- CATTERALL, W. A. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.* 20: 15-43, 1980.
- CRUZ-HÖFLING, M. A. and VITAL BRAZIL, O. Alterações ultraestruturais causadas pela veratrina no diafragma isolado de camundongo. [Abstract] In: *Proceedings of the V Annual Meeting of FESBE*, p. 324, Caxambu, MG, Brasil, 1990.
- FREITAS, E. M. S.; SILVA DAL PAI, M. and CRUZ-HÖFLING, M. A. Mouse *extensor digitorum longus* and *soleus* show distinctive ultrastructural changes induced by veratrine. 2000a. *Exp. Neurol.* (submitted).
- FREITAS, E. M. S.; SILVA DAL PAI, M. and CRUZ-HÖFLING, M. A. A histochemical study of the interaction of mouse *extensor digitorum longus* and *soleus* muscles with veratrine. 2000b. *Exp. Neurol.* (submitted).
- FREITAS, E. M. S.; SILVA DAL PAI, M. and CRUZ-HÖFLING, M. A. Histoenzymological and ultrastructural changes in lateral muscle fibers of *Oreochromis niloticus* (Teleostei: Cichlidae) after local injection of veratrine. 2000c. (submitted)
- HONERJÄGER, P.; DUGAS, M. and ZONG, X-G. Mutually exclusive action of cationic veratridine and cevadine at an intracellular site of the cardiac sodium channel. *J. Gen. Physiol.* 99: 699-720, 1992.
- KOGA, Y.; NONAKA, I.; SUNOHARA, N.; YAMANAKA, R. and KUMAGAI, K. Variability in the activity of respiratory chain enzymes in mitochondrial myopathies. *Acta Neuropathol.* 76: 135-141, 1988.
- LINDAL, S.; LUND, I.; TORBERGSEN, T.; AASLY, J.; MELLGREN, S. I.; BORUD, O. and MONSTAD, P. Mitochondrial diseases and myopathies: a series of muscle biopsy specimens with ultrastructural changes in the mitochondria. *Ultrastruc. Pathol.* 16: 263-275, 1992.

- MELMED, C.; KARPATI, G. and CARPENTER, S. Experimental mitochondrial myopathy produced by *in vitro* uncoupling of oxidative phosphorylation. *J. Neurol. Sci.* **26**: 305-318, 1975.
- NISHINO, I.; KOBAYASHI, O.; GOTO, Y-I; KURIHARA, M.; KUMAGAI, K.; FUJITA, T.; HASHIMOTO, K.; HORAI, S. and NONAKA, I. A new congenital muscular dystrophy with mitochondrial structural abnormalities. *Muscle Nerve* **21**: 40-47, 1998.
- PEMBERTON, K. E.; NGUYEN, M. L.; PRIOR, C.; PARSONS, S. M. and MARSHALL, I. A. Effect of veratridine on miniature endplate current amplitudes at the rat neuromuscular junction and acetylcholine uptake by *Torpedo* synaptic vesicles. *Brain Res.* **671**: 267-274, 1995.
- SHAH, A. J.; SAHGAL, V.; MUSCHLER, G.; SUBRAMANI, V. and SINGH, H. Morphogenesis of the mitochondrial alterations in muscle diseases. *J. Neurol. Sci.* **55**: 25-37, 1982.
- ULBRICHT, W. Effects of veratridine on sodium currents and fluxes. *Rev. Physiol. Biochem. Pharmacol.* **133**: 1-54, 1998.
- VITAL BRAZIL, O. and FONTANA, M. D. Unequal depolarization of the membrane of the rat diaphragm muscle fibers caused by veratrine. *Pflügers Arch.* **404**: 45-49, 1985.

LEGENDS FOR FIGURES

Figure 01 - *Electronmicrographs of mitochondria from mice EDL muscle fibers.*

(A) – Control muscle: mitochondria with typical morphology; (B) – Veratrinized muscle at 15 min: Abnormal swollen mitochondria with disorganized cristae, atypical matrix, outer and inner membrane; (C-E) - At 30 min, various patterns of degenerative changes: huge increase of the mitochondrial dimensions probably owing to fluid collection. Note the electrolucency of matrix and the disarray of cristae which exhibit presence of straight parallel dark lines into the organelle (C), or cristae constituting trabeculae, some of them fused and/or interspersed by enlarged intercristal spaces (D), or ruptured mitochondria showing absence of the intermembranous compartment and marked reduction of cristae surface (E); (F) - At 60 min: the degenerative changes are in a process of recovery. Note that the dimensions of the organelles tend to be restored and there was an increase of the cristae surface. Bar = 0.5 μ m for all panels.

Figure 02 - *Electronmicrographs of mitochondria from SOL muscle fibers.*

(A) Control muscle: mitochondria are elongated and well-provided with cristae, which are immersed in an electrondense matrix; (B-D) Veratrinized muscle at 15 min: the great majority of mitochondria were degenerated. Disruption of the membranes and areas devoid of cristae, which were immersed in an electronlucent matrix were common (B-D). Note that flocculent densities and aberrant cristae also are present (D); (E) At 30 min: the degenerative picture is almost the same, and persists the marked increase of mitochondrial volume paralleled in a number of organelles with the rupture of the outer and inner membrane, as shown in the picture; (F) By 60 min is visible the recovery of mitochondria structure, but their dimensions were not fully restored. Bar= 0.5 μ m for all panels.

Figure 03 - *Figure shows the oxygen consumption in mouse isolated muscle mitochondria.*

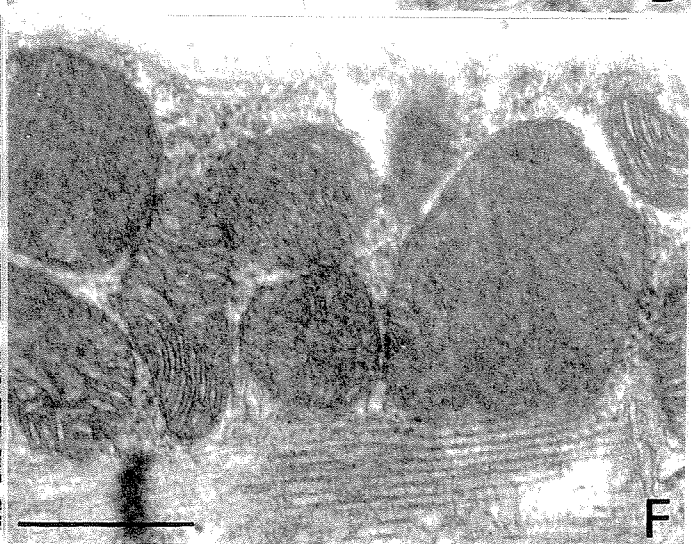
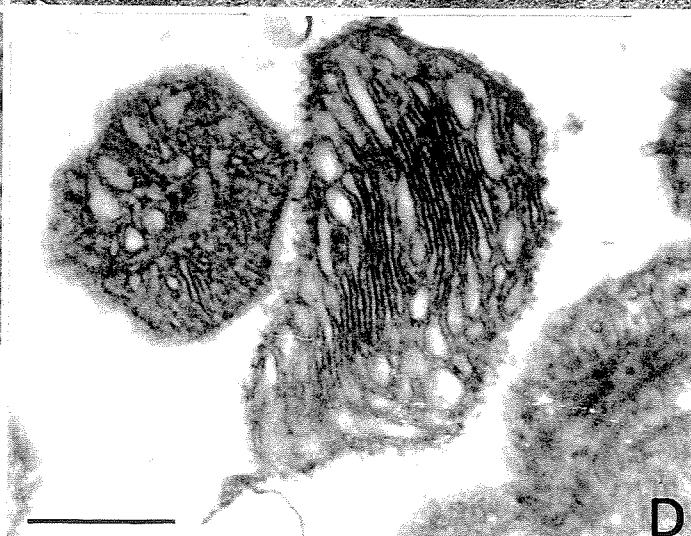
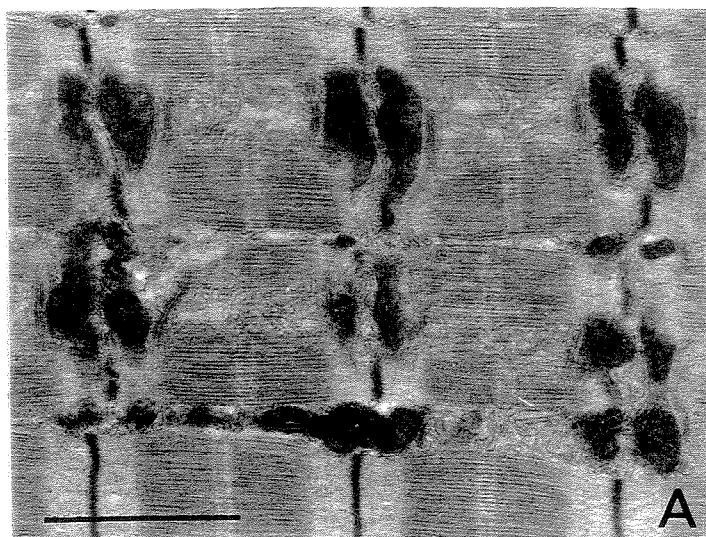
(A) The experiments started with the addition of 3 mM succinate (SUC). State 3 respiration rate was reached by the addition of 200 nmoles ADP. Numbers on the trace refer to oxygen consumption rates of nanoatoms oxygen/min/mg of protein. (B) Mouse muscle mitochondria were incubated in the presence of reaction medium and 10 µg/ml veratrine for 1 min. It was observed a decrease in state 3 respiration rate (from 131 to 101) and an increase in state 4 (from 20 to 55) after addition of muscle mitochondria with veratrine.

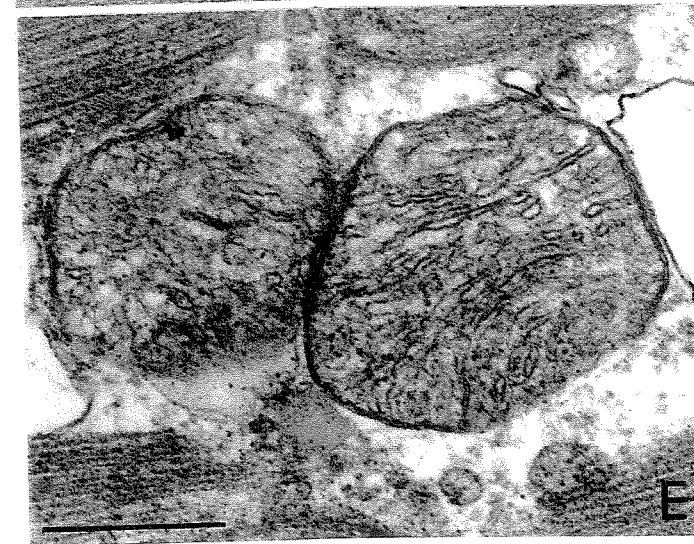
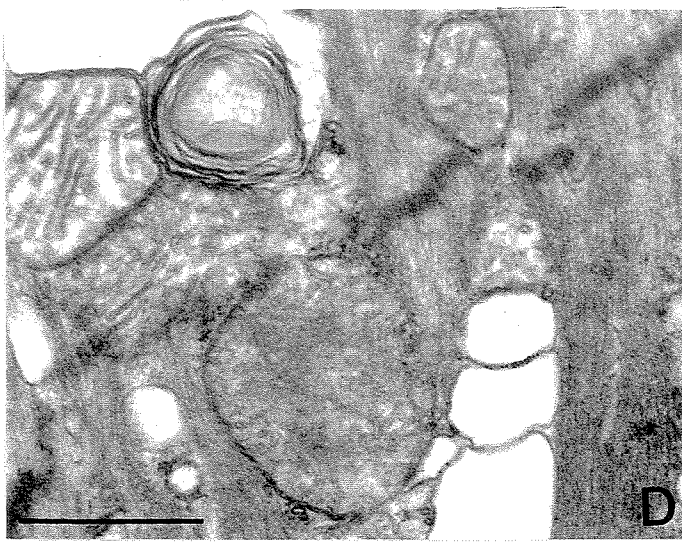
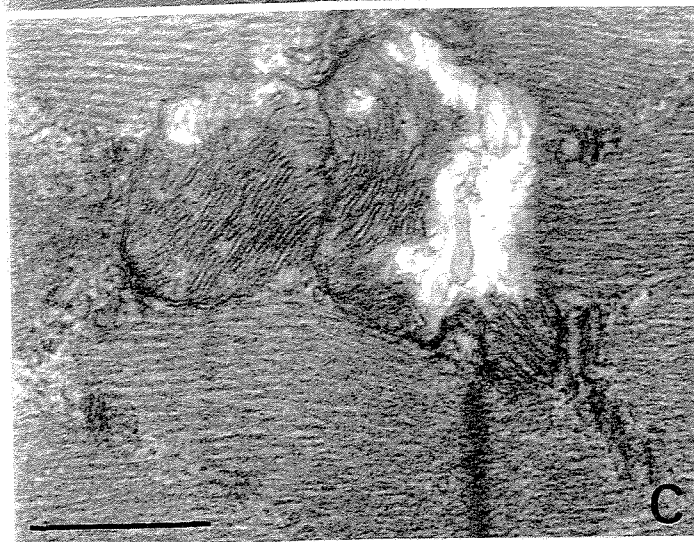
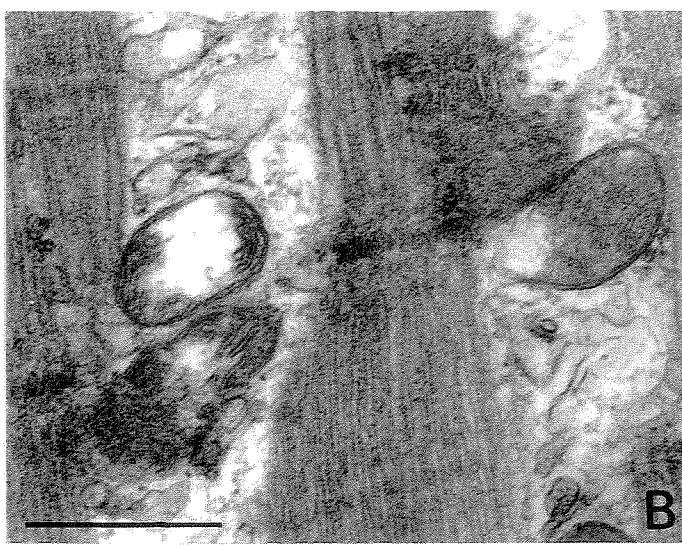
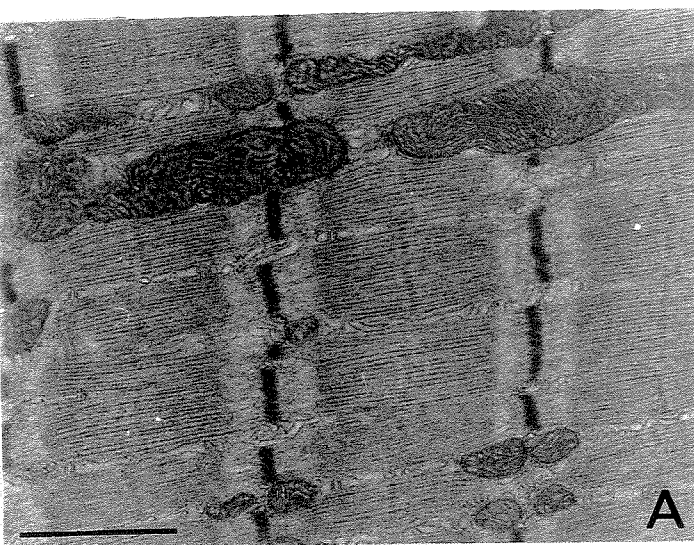
Figure 04 - *Assay conditions as described under “Materials and Methods” for Rats.*

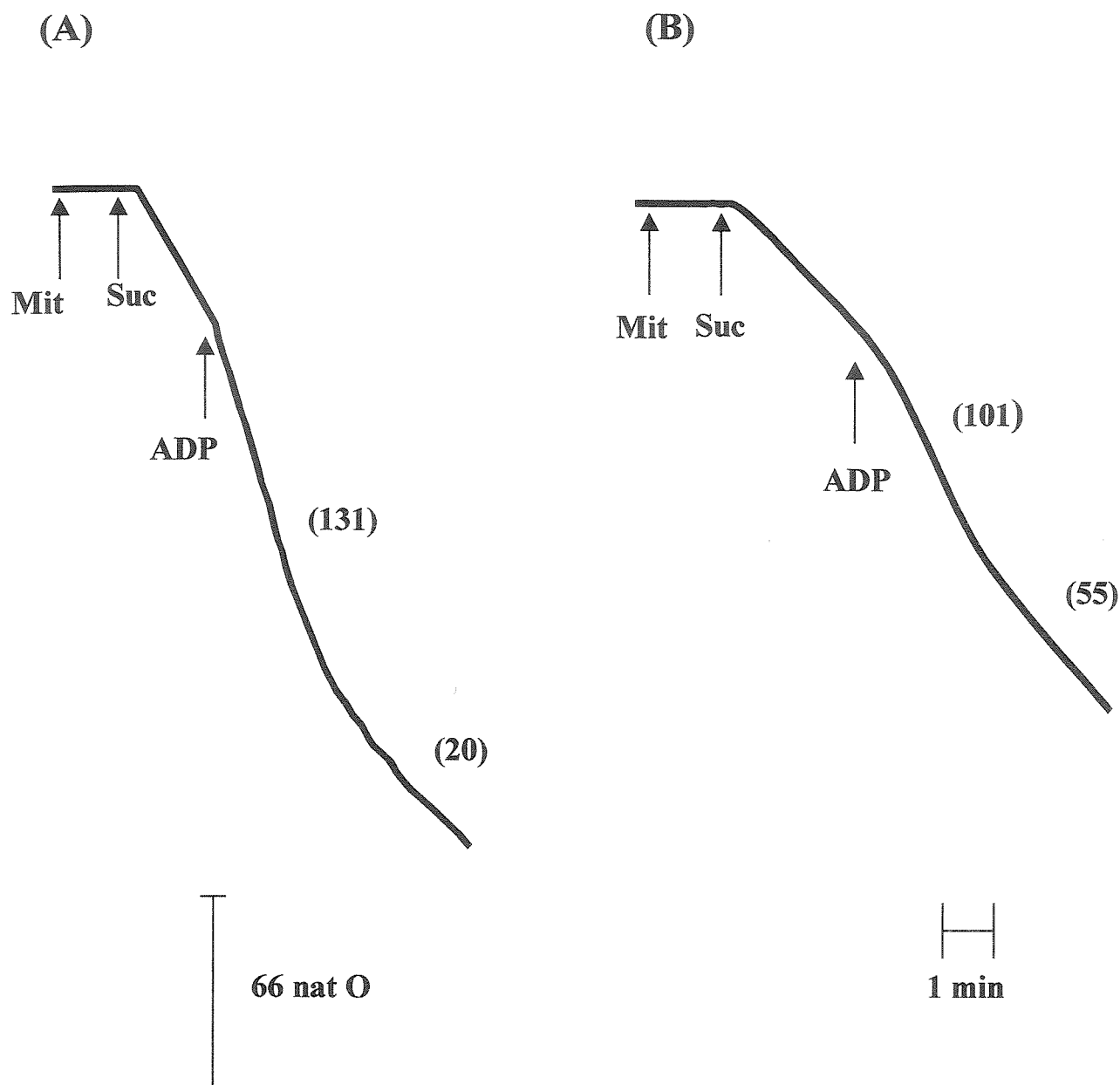
(A) The figure shows the oxygen consumption in isolated rat muscle mitochondria. The experiments started with the addition of 10 mM glutamate (GLUT). State 3 respiration rate, was reached by the addition of 200 nmoles ADP. Numbers on the trace refer to oxygen consumption rates of nanoatoms oxygen/min/mg of protein. (B) Rat muscle mitochondria were incubated in the presence of reaction medium and 50 µg/ml veratrine for 1 min. It was observed an increase in state 3 respiration (from 253 to 396) and 4 (from 44 to 121) after contact with veratrine.

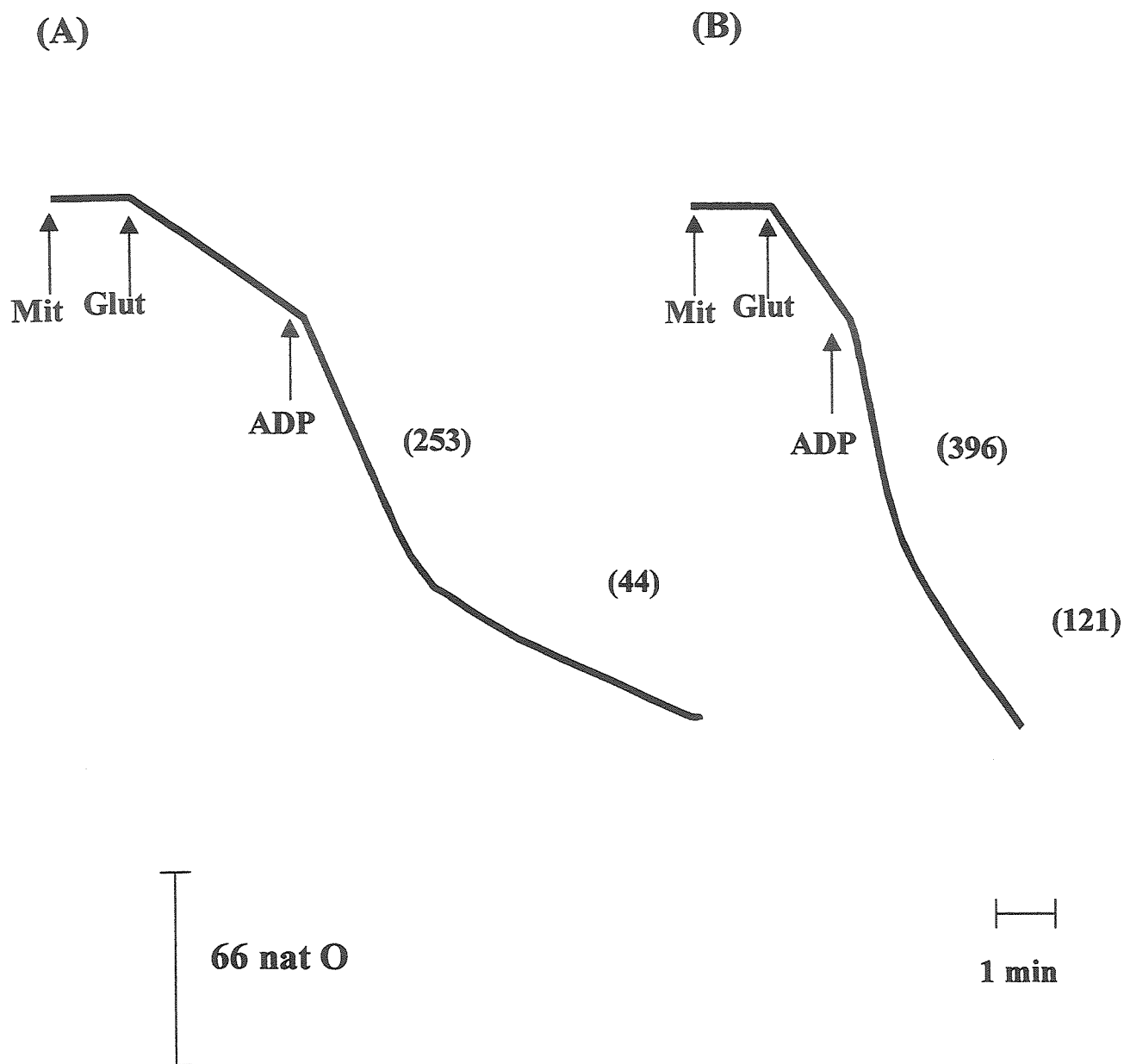
Figure 05 - *The figure shows the oxygen consumption in rat isolated liver mitochondria.*

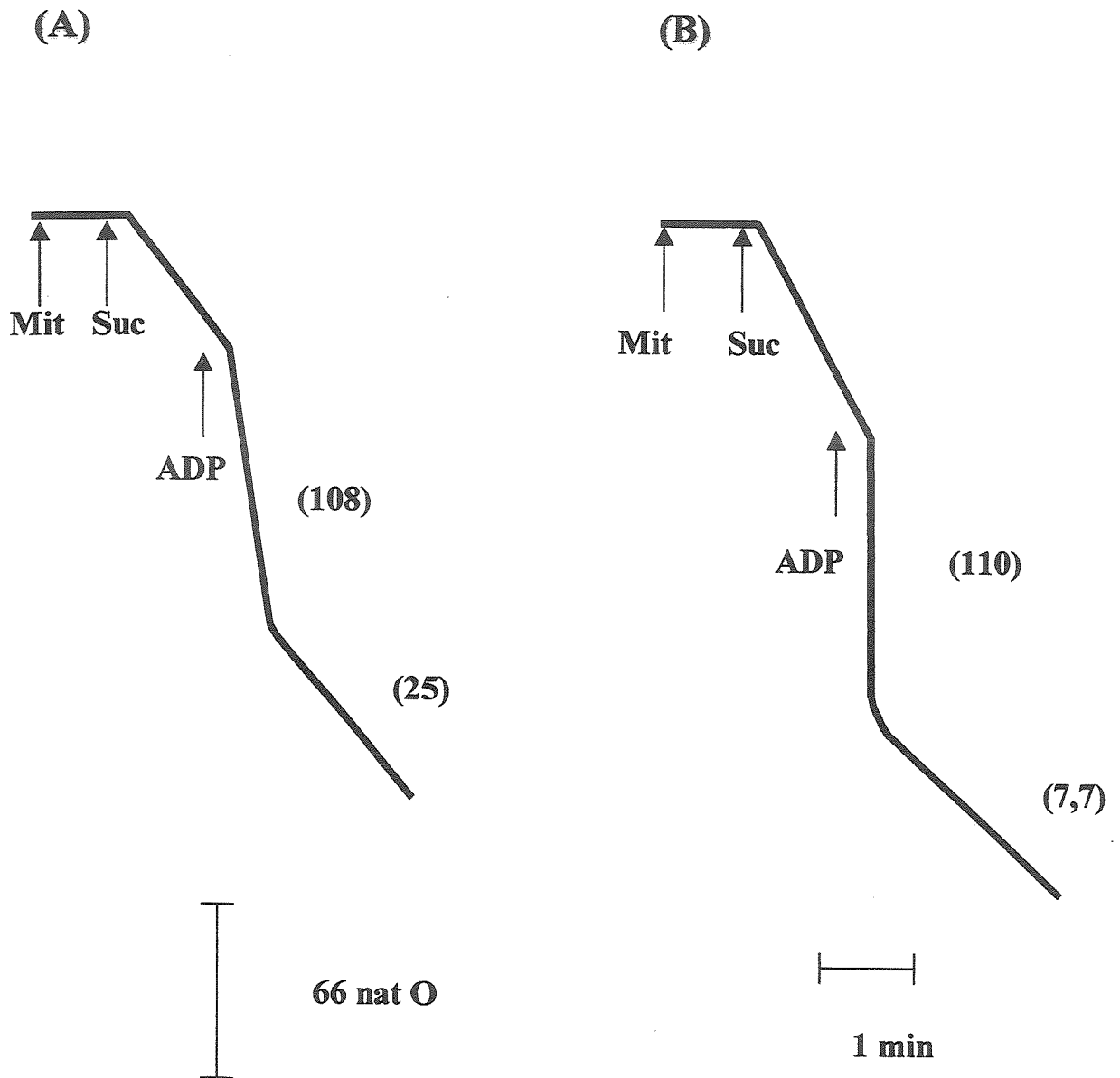
(A) The assays started with the addition of 3 mM succinate (SUC). State 3 respiration rate, was reached by the addition of 200 nmoles ADP. Numbers on the trace refer to oxygen consumption rates of nanoatoms oxygen/min/mg of protein. (B) RLM were incubated (during 1 min) in the presence of reaction medium and veratrine (50 µg/ml). It was observed an increase in state 3 respiration (from 44 to 121) and a decrease in state 4 (from 25 to 7.7) after addition of liver mitochondria with veratrine (50 µg/ml).











CONCLUSÕES

A ação da veratrina é complexa e envolve mecanismos dependentes de fatores locais dependentes de controle gênico. Esta afirmação decorre da observação de que as alterações induzidas pelo alcalóide não se expressam igualmente nos modelos utilizados.

Nós concluímos que a interação da veratrina com as fibras musculares esqueléticas é espécie-específica, músculo-específica e tipo de fibra específica, e que a interação da veratrina com as mitocôndrias é tecido(órgão)-específica.

Atribuímos como principal causa dos efeitos produzidos pela veratrina, principalmente nos camundongos, ao seu papel na ativação dos canais de sódio, já que o pré-tratamento com TTX abole praticamente todos os efeitos. Além disso, o fato das alterações reverterem-se espontaneamente ao final dos 60 minutos de experimentação sugerem fortemente a existência de distúrbios hidroeletrolíticos (mais inerentes a distúrbios em canais iônicos).

Finalmente, uma vez que o retículo sarcoplasmático e as mitocôndrias parecem ser os alvos primordiais de ação da veratrina (e que provavelmente as demais alterações observadas na estrutura e atividade metabólica e contrátil da fibra muscular, sejam decorrentes dessa ação primordial no retículo e mitocôndrias), levantamos a hipótese de que a veratrina interfere também com o metabolismo do cálcio, além do metabolismo do sódio. Não é descartada a possibilidade de entrada da veratrina para o interior da célula.

REFERÊNCIAS BIBLIOGRÁFICAS

- ALBERTS, B. 1994. Músculo. In: ALBERTS, B.; BRAY, D.; LEWIS, J.; RAFF, M.; ROBERTS, K. & WATSON, J. D. ed. - **Biologia Molecular da Célula**. Porto Alegre, Artes Médicas. 1994a. p. 847-858.
- ALBERTS, B. Transporte transmembrana de moléculas pequenas e bases iônicas da excitabilidade da membrana. In: ALBERTS, B.; BRAY, D.; LEWIS, J.; RAFF, M.; ROBERTS, K. & WATSON, J. D. ed. - **Biologia Molecular da Célula**. Porto Alegre, Artes Médicas. 1994b. p. 508-549.
- ALBUQUERQUE, E.; WARNICK, J. E. & SANSONE, F. M. The pharmacology of batrachotoxin. II. Effect on electrical properties of the mammalian nerve and skeletal muscle membranes. **J Pharmac Exp Ther** 176: 511-528, 1971.
- ARMSTRONG, R. B.; SAUBERT, C. W.; SEEHERMAN, H. J. & TAYLOR, C. R. Distribution of fiber types in locomotory muscles of dogs. **Am J Anat** 163: 87-98, 1982.
- ARMSTRONG, R. B. & PHELPS, R. O. Muscle fiber type composition on the rat hindlimb. **Am J Anat** 171: 259-272, 1984.
- BECKER, S. & GORDON, R. D. Sodium channel specific neurotoxins: recent advances in the understanding of their molecular mechanisms. In: HERKEN, H. & HUCHO, F. E. ed. - **Handbook of Experimental Pharmacology. Selective Neurotoxicity**. New York-Berlin, Springer-Verlag. 1992. p.719-737, v. 102.
- BENFORADO, J. M. The veratrum alkaloids. In: ROOT, W. S. & HOFMANN, F. G. ed. **Physiological pharmacology**. New York, Academic Press. 1967. p. 331-398.

- BENDERS, A. A. G. M.; OOSTERHOF, A., WEVERS, R. A. & VEERKAMP, J. H. Excitation-contraction coupling of cultured human skeletal muscle cells and the relation between basal cytosolic Ca^{2+} and excitability. *Cell Calcium* 21: 81-91, 1997.
- BETTO, D. D.; ZERBATO, E. & BETTO, R. Type 1, 2A and 2B myosin heavy chain electrophoretic analysis of rat muscle fibers. *Biochem Biophys Res Commun* 138: 981-987, 1986.
- BROOKE, M. H. & ENGEL, W. K. The histografic analysis for human muscle biopsies with regard to fiber types. 1. Adult male and female. *Neurology* 19: 221-233, 1969a.
- BROOKE, M. H. & ENGEL, W. K. The histografic analysis for human muscle biopsies with regard to fiber types. 4. Children's biopsies. *Neurology* 19: 591-605, 1969b.
- BROOKE, M. H. & KAISER, K. K. Three "myosin adenosine triphosphatase" systems: The nature of their pH hability and sulphydryl dependence. *J Histochem Cytochem* 18: 670-672, 1970.
- BROWNLOW, H.; WHITMORE, I. & WILLAN, P. L. T. A quantitative study of the histochemical and morphometric characteristics of the human *cricopharyngeus* muscle. *J Anat* 166: 67-75, 1989.
- CALDWELL, J. H.; CAMPBELL, D. T. & BEAM, K. G. Na^+ channel distribution in vertebrate skeletal muscle. *J Gen Physiol* 87: 907-932, 1986.
- CAMERON, D. L. & TU, A. T. Chemical and functional homology of myotoxin *a* from prairie rattlesnake venom and crotamine from South American rattlesnake venom. *Biochim Biophys Acta* 532: 147-154, 1978.

- CAPETANAKI, Y.; MILNER, D. J. & WEITZER, G. Desmin in muscle formation and maintenance: knockouts and consequence. *Cell Struct Func* 22: 103-116, 1997.
- CATTERALL, W. A. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu Rev Pharmacol Toxicol* 20: 15-43, 1980.
- CATTERALL, W. A. Structure and modulation of Na⁺ and Ca²⁺ channels. *Ann N Y Acad Sci* 707: 1-19, 1993.
- CHANG, C. C. & TSENG, H. K. Effect of crotoxin, a toxin of South American rattlesnake venom, on the sodium channel of murine skeletal muscle. *Br J Pharmacol* 63: 551-559, 1978.
- CROSBY, D. G. Minor insecticides of plant origin. In: JACOBSON, M. J. & CROSBY, D. G. ed. *Naturally occurring insecticides*. New York, Dekker. 1971, p. 177-239.
- CROW, M. T. & KUSHMERICK, M. J. Chemical energetics of slow- and fast-twitch muscles of the mouse. *J Gen Physiol* 79: 147-166, 1982.
- CRUZ-HÖFLING, M. A.; LOVE, S.; BROOK, G. & DUCHEN, L. W. Effects of *Phoneutria nigriventer* spider venom on mouse peripheral nerve. *Quart J Exp Physiol* 70: 623-640, 1985.
- CRUZ-HÖFLING, M. A.; NEDER, F.; RODRIGUES-SIMIONI, L. & VITAL BRAZIL, O. Effects of veratrine on the ultrastructure of nerve terminals of the mouse diaphragm *in vitro*. [Abstract] In: *Anais do XI Colóquio da Sociedade Brasileira de Microscopia Eletrônica*. pp. 139. Caxambu, MG, Brasil. 1987.
- CRUZ-HÖFLING, M. A. & VITAL BRAZIL, O. Alterações ultraestruturais causadas pela veratrina no diafragma isolado de camundongo. [Abstract] In: *Anais do V Encontro Anual da FESBE*. pp. 324. Caxambu, MG, Brasil. 1990.

- DUBOWITZ, V. & BROOKE, M. In: DUBOWITZ, V. & BROOKE, M. ed. - **Muscle biopsy: A Modern Approach**. London, Saunders. 1973, p. 475.
- DUBOWITZ, V. In: DUBOWITZ, V. ed. - **Muscle Biopsy: A Practical Approach**. London, Bailliere Tindall. 1985, p. 720.
- EASTWOOD, A. B.; FRANZINI-ARMSTRONG, C. & PERACCHIA, C. Structure of membranes in crayfish muscle: Comparison of phasic and tonic fibers. **J Muscle Res Cell Motil** 3: 273-294, 1982.
- EGGINTON, S. & JOHNSTON, I. A. A morphometric analysis of regional differences in myotomal muscle ultrastructure in the juvenile eel. **Cell Tissue Res** 222: 579-596, 1982.
- ESBÉRARD, C. A. O músculo. In: AIRES, M. M. ed - **Fisiologia**. Rio de Janeiro, Guanabara Koogan,. 1991, p. 60-89,.
- FLORENDO, J. A.; REGER, J. F. & LAW, P. K. Electrophysiologic differences between mouse *extensor digitorum longus* and *soleus*. **Exp Neurol** 82: 404-412, 1983.
- FLUCHER, B. E. Structural analysis of muscle development: transverse tubules, sarcoplasmic reticulum and triad. **Dev Biol** 154: 245-260, 1992.
- FAVREAU, P.; KRINM, I.; LEGALL, F.; BOBENRIETH, M.J.; LAMTHANH, H.; BOVET, F.; SERVENT, D.; MOLGO, J.; MENEZ, A.; LETOURNEIX, Y. & LANCELIN, J. M. Biochemical characterization and nuclear magnetic resonance structure of novel alpha-conotoxins isolated from the venom of *Conus consors*. **Biochemistry** 38: 6317-6326, 1999.

- FUHRMAN, F. A. Tetrodotoxin, Tarichatoxin and Chiriquitoxin: Historical perspectives. *Ann N Y Acad Sci* 479: 1-14, 1986.
- GEORGE, J. C. & BOKADAWALA, F. D. Cellular organization and fat utilization in fish muscle. *J Am Morphol Physiol* 11: 124-132, 1964.
- GORZA, L. Identification of a novel type 2 fiber population in mammalian skeletal muscle by combined use of histochemical myosin ATPase and anti-myosin monoclonal antibodies. *J Histochem Cytochem* 38: 257-265, 1990.
- HARRIS, J. B. & CULLEN, M. J. Muscle necrosis caused by snake venoms and toxins. *Electron Microsc Rev* 3: 183-211, 1990.
- HILLE, B. & CAMPBELL, D. T. An improved vaseline gap voltage clamp for skeletal muscle fibers. *J Gen Physiol* 67: 265-293, 1976.
- HODGKIN, A. L. & HUXLEY, A. F. The components of membrane conductance in the giant axon of *Loligo*. *J Physiol* 116: 473-496, 1952.
- HONERJÄGER, P. Cardioactive substances that prolong the open state of sodium channels. *Rev Physiol Biochem Pharmacol* 92: 1-74, 1982.
- HONERJÄGER, P.; DUGAS, M. & ZONG, X-G. Mutually exclusive action of cationic veratridine and cevadine at an intracellular site of the cardiac sodium channel. *J Gen Physiol* 99: 699-720, 1992.
- HONG, S. J. & CHANG, C. C. Eletrophysiological studies of myotoxin α , isolated from prairie rattlesnake (*Crotalus viridis viridis*) venom, on murine skeletal muscles. *Toxicon* 23: 927-937, 1985.

- HORACKOVA, M. & VASSORT, G. Ionic mechanism of inotropic effect of veratridine on frog heart. *Pfugers Arch* 341: 281-284, 1973.
- HOYLE, J.; GILL, H. S. & WEATHERLEY, A. H. Histochemical characterization of myotomal muscle in grass pickerel, *Esox americanus vermiculatus* and the muskelunge, *Esox masquinongy*. *J Fish Biol* 28: 393-401, 1986.
- JANSEN, J. K. & FLADBY, T. The perinatal reorganization of the innervation of skeletal muscle in mammals. *Progr Neurobiol* 34: 39-90, 1990.
- KRAYER, O. & ACHESON, G. H. The pharmacology of the veratrum alkaloids. *Physiol Rev* 26:383-446, 1946.
- KOHLHAUPT, P. In: HELMUT, G. & KING, M. ed - *Fleurs des Alpes*. Paris, Hatier. 1966. p.45-46. v. I.
- LAZDUNSKI, M & RENAUD, J F. The action of cardiotoxins on the cardiac plasma membranes. *Annu Rev Physiol* 44: 463-473, 1982.
- LEHOUELLEUR, J.; LASZZYCA, P. & BRUNES, J. Sodium-dependent depolarizing potentials in veratrinized crayfish muscle fibres. *Neurosci Lett* 89: 299-304, 1988.
- LI, Z.; MERICKSKAY, M.; AGBULUT, O.; BUTLER-BROWNE, G.; CARLSSON, L.; THORNELL, L. E.; BABINET, C. & PAULIN, D. Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. *J Cell Biol* 139: 129-144, 1997.
- LINKE, W. A.; IVEMEYER, M.; OLIVIERI, N.; KOLMERER, B.; RÜEGG, J. C. & LABEIT, S. Towards a molecular understanding of the elasticity of titin. *J Mol Biol* 261: 62-71, 1996.

- LOVE, S.; CRUZ-HÖFLING, M. A. & DUCHEN, L. W. Morphological abnormalities in myelinated nerve fibres caused by *Leiurus*, *Centruroides* and *Phoneutria* venoms and their prevention by tetrodotoxin. *Quart J Exp Physiol* 71: 115-122, 1986.
- MACFARLANE, W. V. & MEARS, J. D. Chemical modification of intracellularly recorded after-potentials of frog skeletal muscle. *J Physiol* 142: 78-96, 1958.
- MAHON, M.; TOMAN, A.; WILLAN, P. L T. & BAGNALL, K. M. Variability of histochemical and morphometric data from needle biopsy specimens of human *quadriceps femoris* muscle. *J Neurol Sci* 63: 85-100, 1984.
- MASCARELLO, F.; ROMANELLO, M. G. & SCAPOLO, P. A. Histochemical and immunohistochemical profile of pink muscle fibres in some teleosts. *Histochemistry* 84: 251-255, 1986.
- MATAVEL, A. C.; FERREIRA-ALVES, D. L.; BEIRAO, P. S. & CRUZ, J. S. Tension generation and increase in voltage-activated Na^+ current by crotamine. *Eur J Pharmacol* 348: 167-173, 1998.
- MATTEI, C.; MOLGO, J.; LEGRAND, A. M. & BENOIT, E. [Ciguatoxins and brevetoxins: dissection of the neurobiological actions]. *J Soc Biol* 193: 329-344, 1999.
- MATTIELLO-SVERZUT, A. C. & CRUZ-HÖFLING, M. A. Toxin 2 (PhTx2), a neurotoxic fraction from *Phoneutria nigriventer* spider venom, causes acute morphological changes in mouse skeletal muscle. *Toxicon* 38: 793-812, 2000.
- MILTON, R. L. & BEHFOROUZ, M. A. Na^+ channel density in extra junctional sarcolemma of fast and slow twitch mouse skeletal muscle fibers: functional implications and plasticity after fast motoneuron transplantation to a slow muscle. *J Muscle Res Cell Motil* 16: 430-439, 1995.

- NACHLAS, M. M.; TSOU, K. C.; SOUZA, E.; CHING, C. S. & ELIGMAN, A. M. Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. *J Histochem Cytochem* 5: 420, 1957.
- NÁNÁSI, P. P.; VARRO, A.; LATHROP, D. A. & BRYONT, S. H. Effects of veratridine on Na^+ and Ca^{2+} currents in frog skeletal muscle. *Gen Pharmacol* 25: 1661-1666, 1994.
- NEMETH, P. M.; PETTER, D. & VRBOVÁ, G. Comparison of enzyme activities among single muscle fibres within defined motor units. *J Physiol* 311: 489-495, 1981.
- NODA, M.; SHIMIZU, S.; TANABE, T.; TAKAI, T.; KAYANO, T.; IKEDA, T.; TAKAHASHI, H.; NAKAYAMA, H.; KANOKA, Y.; MINAMINO, N.; KANGAWA, K.; MATSUO, H.; RAFTERY, M. A.; HIROSE, T.; INAYAMA, S.; HAYASHIDA, H.; MIYATA, T. & NUMA, S. Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. *Nature* 312: 121-127, 1984.
- NODA, M. Structure and function of sodium channels. *Ann N Y Acad Sci* 707: 20-37, 1993.
- OGATA, T. A histochemical study of the red and white muscle fibres. III. Activity of the diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase in muscle fibres. *Acta Med Okayama* 12: 233-240, 1958.
- OGATA, T. & MORI, M. Histochemical study of oxydative enzymes in vertebrate muscles. *J Histochem Cytochem* 12: 171-182, 1964.
- OGATA, T. Morphological and cytochemical features of fiber types in vertebrate skeletal muscle. *CRC Crit Rev Anat Cell Biol* 1: 229-275, 1988.

- OWNBY, C. L.; CAMERON, D. & TU, A. T. Isolation of myotoxic component from rattlesnake (*Crotalus viridis viridis*) venom. *Am J Pathol* **85**: 149-166, 1976.
- PETER, J. B.; BARNARD, R. J.; EDGERTON, U. R.; GILLESPIE, C. A. & STEMPEL, K E. Metabolic profiles of three types of fibers of skeletal muscles in guinea pig and rabbits. *Biochemistry* **11**: 2627-2633, 1972.
- PETTE, D. & STARON, R. S. Cellular and molecular diversities of mammalian skeletal muscle fibers. *Rev Physiol Biochem Pharmacol* **116**: 2-47, 1990.
- PETTE, D. & STARON, R. S. Mammalian skeletal muscle fiber type transitions. *Int Rev Cytol* **170**: 143-223, 1997.
- PIEROBON-BORMIOLI, S.; SARTORE, S.; DALLA LIBERA, L.; VITADELLO, M. & SCHIAFFINO, S. "Fast" isomyosins and fiber types in mammalian skeletal muscle. *J Histochem Cytochem* **29**:1179-1188, 1981.
- RITCHIE, J. M. & ROGART, R. B. Density of sodium channels in mammalian myelinated nerve fibers and nature of the axonal membrane under the myelin sheath. *Proc Natl Acad Sci USA* **74**: 211-215, 1977.
- RITCHIE, J. M. & CHIU, Y. Distribution of sodium and potassium channels in mammalian myelinated nerve. *Adv Neurol* **31**: 329-342, 1981.
- ROSEMBLATT, M.; HIDALGO, C.; VERGANA, C. & IKEMOTO, N. Immunological and biochemical properties of transverse tubule membranes isolated from rabbit skeletal muscle. *J Biol Chem* **256**: 8140-8148, 1981.
- ROSS, M. H. & ROWRELL, L. J. O tecido muscular. In: ROSS, M. H. & ROWRELL, L. J. ed. – *Histologia*. São Paulo, Panamericana. 1993. p.203-216.

- RUFF, R. L. Na^+ current density at and away from end plates on rat fast- and slow-twitch skeletal muscle fibers. *Am J Physiol* 262: C229-C234, 1992.
- SALGADO, V. L.; YEH, J. Z. & NARAHASHI, T. Use- and voltage-dependent block of the sodium channel by saxitoxin. *Ann N Y Acad Sci* 479: 84-95, 1986.
- SECKEL, B. R. Enhancement of peripheral nerve regeneration. *Muscle Nerve* 13: 785-800, 1990.
- SCHIAFFINO, S.; GORZA, L.; DONES, I.; CORNELIO, F. & SARTORE, S. Fetal myosin immunoreactivity in human dystrophic muscle. *Muscle Nerve* 9: 51-58, 1986.
- SHIROKOVA, N.; GARCÍA, J.; PIZARRO, G. & RÍOS, E. Ca^{2+} release from the sarcoplasmic reticulum compared in Amphibian and Mammalian skeletal muscle. *J Gen Physiol* 107: 1-18, 1996.
- SUTRO, J. B. Kinetics of veratridine action on Na^+ channels of skeletal muscle. *J Gen Physiol* 87: 1-24, 1986.
- STARON, R. S. & PETTE, D. Correlation between myofibrillar ATPase activity and myosin heavy chain composition in rabbit muscle fibers. *Histochemistry* 86: 19-23, 1986.
- STEIN, J. M. & PADYKULA, H. A. Histochemical classification of individual skeletal muscle fibres of the rat. *Am J Anat* 110: 103-123, 1962.
- STRYER, L. Fosforilação Oxidativa. In: STRYER, L. ed. – *Bioquímica*. Rio de Janeiro, Guanabara Koogan. 1996. p. 501-528.

- SQUIRE, J. M. Architecture and function in the muscle sarcomere. *Curr Opin Struct Biol* 7: 247-257, 1997.
- TE KRONIE, G.; TATARCZUCH, L. & RAAMSDONK, W. Muscle fibre types in the myotome of stickleback, *Gasterosteus aculeatus* L.; a histochemical immunohistochemical and ultrastructural study. *J Fish Biol* 22: 303-316, 1983.
- ULBRICHT, W. Voltage clamp studies of veratrinized frog nodes. *J Cell Comp Physiol* 66: 91-98, 1965.
- ULBRICHT, W. The effect of veratridine on excitable membranes of nerve and muscle. *Ergeb Physiol Biol Exp Pharmacol* 61: 18-71, 1969.
- ULBRICHT, W. Effects of veratridine on sodium currents and fluxes. *Rev Physiol Biochem Pharmacol* 133: 1-54, 1998.
- VITAL BRAZIL, O. & FONTANA, M. D. Unequal depolarization of the membrane of the rat diaphragm muscle fibres caused by veratrine. *Pflügers Arch* 404: 45-49, 1985.
- VITAL BRAZIL, O. Farmacologia do canal do sódio: modo de ação de toxinas. *Ciência e Cultura* 38: 324-328, 1986.
- VITAL BRAZIL, O. & FONTANA, M. D. Toxins as tools in the study of sodium channel distribution in the muscle fibre membrane. *Toxicon* 31: 1085-1098, 1993.
- VOLPE, P.; DAMIANI, E. MAURER, A. & TU, A. T. Interaction of myotoxin α with the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum. *Arch Biochem Biophys* 246: 90-97, 1986.

- WALESBY, N. J. & JOHNSTON, I. A. Fibre types in locomotory muscles of an Antarctic teleost *Nottothenia rossii*. A histochemical, ultrastructural and biochemical study. *Cell Tissue Res* 208: 143-164, 1980.
- WARNICK, J. E.; ALBUQUERQUE, E. X. & SANSONE, F. M. The pharmacology of batrachotoxin. I. Effects on the contractile mechanism and on neuromuscular transmission of mammalian skeletal muscle. *J Pharmacol Exp Ther* 176: 497-510, 1971.
- WAYNE MOORE, G. R.; BOEGMAN, R. J.; ROBERTSON, D. M. & RAINE, C. S. Acute stages of batrachotoxin-induced neuropathy: a morphologic study of a sodium-channel toxin. *J Neurocytol* 15: 573-583, 1986.
- WEATHERLEY, A. & GILL, H. S. Growth increase produced by bovine growth hormone in grass pickerel, *Esox americanus vermiculatus*, and the underlying dynamics of muscle fibre growth. *Aquaculture* 65: 55-66, 1987.
- ZARDINI, D. M. & PARRY, D. J. Identification, distribution, and myosin subunit composition of type IIX fibers in mouse muscles. *Muscle Nerve* 17: 1308-1316, 1994.

