UNIVERSIDADE ESTADUAL DE CAMPINAS

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AÇÃO TÓXICA DA VERATRINA EM MITOCÔNDRIAS E NO RETÍCULO SARCOPLASMÁTICO

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

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"Pedras no caminho? Guardo todas, um dia vou construir um castelo...."

Fernando Pessoa

RESUMO

A veratrina, uma mistura de alcalóides obtidos da espécie de planta *Shoenocaulon officinale*, é uma toxina lipossolúvel que possui como sítio de ação primária os canais de sódio-dependentes de voltagem. Recentemente, nossos estudos mostraram que a veratrina pode causar mionecrose e as evidências sugerem que as mitocôndrias e o retículo sarcoplasmático (RS) possam ser os alvos intracelulares da ação da veratrina. O objetivo deste trabalho foi investigar os efeitos tóxicos induzidos por diferentes concentrações de veratrina sobre o consumo de oxigênio, a atividade dos complexos da cadeia respiratória e a ultraestrutura das mitocôndrias através de análises bioquímicas, citoquímicas e morfométricas e de microscopia eletrônica de transmissão. Além disso, investigar se a interferência na fisiologia dos canais de sódio induzida pela veratrina altera a ultraestrutura do RS em diferentes tipos de fibras musculares esqueléticas.

Alterações ultraestruturais foram observadas nas mitocôndrias de músculos esqueléticos após a injeção intramuscular ou incubação com veratrina. A mistura de alcalóides diminuiu a atividade das enzimas da cadeia respiratória nicotinamida dinucleotídeo desidrogenase (NADH), succinato desidrogenase (SDH) e citocromo oxidase (COX). Além disso, inibiu significativamente a velocidade 3 da respiração, o controle respiratório (RCR) e a razão ADP/O de mitocôndrias isoladas de músculos esqueléticos de rato, enquanto a velocidade 4 da respiração não foi afetada. Diferentemente, o consumo de oxigênio não foi afetado pela veratrina em mitocôndrias isoladas de fígado. Uma tendência no aumento do diâmetro das mitocôndrias isoladas de músculos esqueléticos foi observada apenas com a concentração de 250 µg/ml de veratrina.

A veratridina, uma toxina isolada presente na composição da mistura veratrina, causou ação tóxica semelhante na respiração mitocondrial.

Também foi observada uma dissipação do potencial elétrico da membrana mitocondrial após a adição de 250 µg/ml de veratrina, entretanto este efeito foi totalmente abolido após a adição de ATP, sugerindo que a ATP sintase não foi afetada pela ação da mistura.

A veratrina também induziu a dilatação das cisternas terminais do RS, desorganização dos sarcômeros e dissociação de miofibrilas em preparações de nervo frênico-diafragma e músculos sóleo e extensor longo dos dedos (EDL) de camundongos e na musculatura lateral de peixes tilápias. Estas alterações morfológicas foram tempo-dependentes e foram antagonizadas pelo bloqueador de canais de sódio, a tetrodotoxina (TTX). Embora as alterações induzidas tenham em comum a grande dilatação das cisternas terminais em todos os músculos analisados, houve uma distinção em relação ao diafragma e músculo lateral da tilápia, que tiveram o aparecimento das alterações mais tardiamente do que o EDL e o sóleo. Por outro lado, o sóleo e o músculo lateral da tilápia apresentaram recuperação parcial das alterações mais precocemente. O diafragma, diferentemente dos músculos periféricos aqui estudados, não mostrou alterações nas mitocôndrias. Os estudos miográficos mostraram que a veratrina induz contrações musculares intensas e prolongadas após estimulação elétrica direta, e bloqueio da condução nervosa após estimulação indireta, dependente da concentração e tempo de incubação.

Nós concluímos que a veratrina e a veratridina provavelmente atuam sobre a configuração fosfolipídica da membrana mitocondrial, explicando as alterações bioquímicas e morfológicas observadas em nosso estudo. Além disso, os resultados sugerem que os efeitos tóxicos destes alcalóides são população mitocondrial-específicos sobre os complexos enzimáticos da cadeia respiratória, uma vez que sua ação é distinta nas mitocôndrias de fígado e músculo esquelético.

Em relação às alterações ultraestruturais vistas no RS e no sarcômero, provavelmente o aumento do influxo de íons sódio induzido pela veratrina altera a fisiologia dos canais iônicos presentes na membrana do RS, alterando conseqüentemente a concentração intracelular de cálcio e a homeostasia da fibra muscular. Vários mecanismos podem estar implicados, desde envolvendo a bomba Na^{+}/K^{+}, a perda de seletividade do canal de sódio após ativação, a alteração do funcionamento do trocador Na^{+}/Ca^{+2}, dentre outros.

O presente trabalho mostra que os alcalóides do *Veratrum* podem ser utilizados não apenas como ferramentas farmacológicas para estudo de canais iônicos, mas também em modelos experimentais de miopatias mitocondriais e associadas à disfunção do retículo sarcoplasmático.

ABSTRACT

The veratrine, a mixture of alkaloids obtained from plant *Schoenocaulon officinale*, is a lipid soluble toxin, which target voltage-gated Na+ channels for their primary action site. Recently, our studies showed that the veratrine may cause myonecrosis and evidences suggested mitochondria and sarcoplasmic reticulum (SR) as intracellular cell targets. The aim of this work was to investigate the effects caused by variable concentration of veratrine on mitochondrial oxygen consumption, respiratory chain enzymes activities and ultrastructure, combining electron microscopy with biochemical, cytochemical and morphometrical analysis. Moreover, we investigated whether the interference on physiology of sodium channels by veratrine it alters the ultrastructure of SR on different types of muscles.

Ultrastructural changes were observed on skeletal muscle mitochondria after intramuscular injection or incubation with veratrine. The mixture of alkaloids decreased mitochondrial nicotinamide adenine dinucleotide dehydrogenase (NADH), succinic dehydrogenase (SDH) and cytochrome oxidase (COX) activities, inhibited significantly and dose-dependently the state 3 respiration rate, respiratory control ratio (RCR) and ADP/O on isolated rat skeletal muscle mitochondria, whereas state 4 was unaffected. Differently, the oxygen consumption on isolated rat liver mitochondria was not affected by veratrine. A tendency of increase in isolated mitochondria diameter from skeletal muscle was seen only with 250 μg/ml veratrine.

Veratridine, a fraction from veratrine mixture, provoked a similar toxic action on mitochondrial respiration.

Dissipation of mitochondrial electrical membrane potential during succinate oxidation was observed upon addition of 250 μg/ml veratrine in isolated rat skeletal muscle mitochondria, nevertheless this effect was totally reversible after addition of ATP, suggesting that the ATP synthase was not affected by the mixture.

Veratrine also induced the dilatation of SR terminal cisternae, disorganization of sarcomeres and dissociation of myofibrils on mouse phrenic nerve-diaphragm muscle preparation soleus and *extensor digitorum longus* (EDL) muscles, and lateral musculature from *Oreochromis niloticus* fish. These morphological changes were time-dependents and antagonized by preatreatment with tetrodotoxin (TTX), a sodium channel blocker. Although the swelling of SR terminal cisternae were observed in all muscles examined, the appearance of alterations in diaphragm and tilapia lateral musculature was delayed in relation to EDL and *soleus*. Yet, the diaphragm, differently from the other peripherical muscles examined did not show mitochondria with damage. On the other hand, *soleus* and fish lateral muscle presented attenuated alterations after 60 min veratrine treatment, indicating a partial reversion of the effects. The myographic record shows that the veratrine causes intense and prolonged contractions after direct electrical stimulation and nerve conduction blockade after indirect stimulation, which were time and concentration-dependent.

We conclude that the veratrine and veratridine would probably act on mitochondrial membrane phospholipid configuration, what would explain the biochemical and morphological changes observed. Moreover, the results suggest that there are mitochondrial population-specific toxic effects of veratrine and veratridine on respiratory chain complexes of mitochondria. In relation to the ultrastructural changes seen on SR and sarcomeres, the increase of Na^+ influx induced by veratrine probably alters the physiology of ionic channels present on SR membranes,

consequently affecting the intracellular calcium concentration and homeostasis of muscle fibers. Several mechanisms could be involved in these alterations as Na^+/K^+ pump, loss of selectivity of sodium channel after activation caused by modification of the gating system, alteration of mechanism of Na^+/Ca^{2+} exchanger, among others.

The present work show that *Veratrum* alkaloids can be used not only as a pharmacological tool for studying mechanisms of sodium channels functioning, but rather can also be utilized inexperimental models of skeletal mitochondrial myopathies and SR dysfunction associated disorders.

SUMÁRIO

1. INTRODUÇÃO

1.1. A veratrina

1.1.1. Composição química

De acordo com a definição dada pela Farmacopéia Britânica (1885), a veratrina é uma mistura de alcalóides, extraída de uma série de plantas do gênero *Veratrum*, pertencentes à família Liliaceae. Dentre elas está a espécie *Schoenocaulon officinale* A. Gray (1840) (cebadilla ou sabadilla), planta nativa dos Andes mexicano (Figura 1) de cujas sementes é extraído o alcalóide. As plantas do gênero *Veratrum* crescem nos Estados Unidos, no Canadá, México e América Central.

Figura 1: A - Exemplar de *S. officinale*, coleção do herbário de La Cruz, Guanacasta, Costa Rica ; **B** - Habitat da espécie: Aragua, Venezuela (Foto: Thomas B. Croat).
Imagens obtidas em http://www.illustratedgarden.org

A mistura é composta principalmente pelos alcalóides cevadina e veratridina, e também pela cevacina, veracevina e vaniloilveracevina (Benforado, 1967). A veratridina é o principal alcalóide e com maior potencial tóxico que compõe esta mistura (Caterall, 1980; Lazdunski & Renaud, 1982). Estes alcalóides são compostos policíclicos, lipossolúveis, com estrutura química esteróide contendo um átomo de nitrogênio secundário ou terciário dentro do anel (Catterall, 1980) (Figura 2).

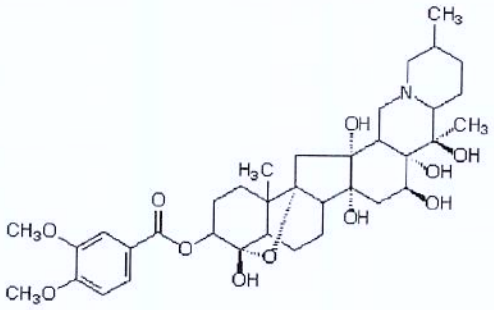

Figure 2: Estrutura química da veratridina

1.1.2. Mecanismo de ação

Estudos fisiológicos e farmacológicos mostram que a veratrina causa a ativação persistente dos canais de sódio-dependentes de voltagem (*cf.* Catterall, 1980), induzindo despolarização e respostas elétricas iterativas em membranas de fibras excitáveis (Vital Brazil & Fontana,1985).

A veratrina, como também outras toxinas lipossolúveis conhecidas como ativadores de canais de Na^+, ligam-se ao sítio 2 dos canais, localizado na região hidrofóbica da membrana celular, interagindo com as hélices transmembrana do canal de Na^+ (Becker & Gordon, 1992) (Figura 3).

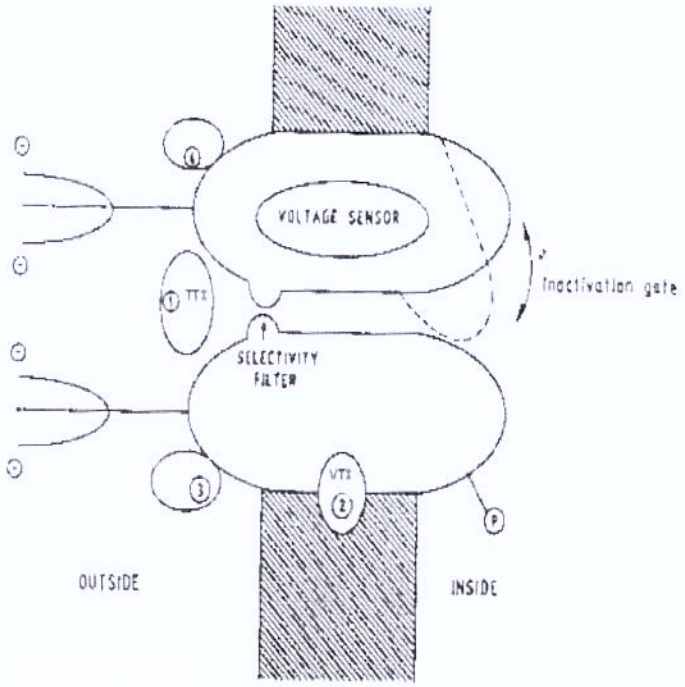

Figura 3: Representação dos sítios de ação da veratrina (VTX) e da tetrodotoxina (TTX) esquema modificado de Becker & Gordon (1992)

Os canais de Na^+-dependentes de voltagem em mamíferos contém uma grande subunidade α, que possui quatro domínios homólogos (D1-D4) com seis segmentos transmembrana (S1-S6), e duas pequenas subunidades β (β1 e β2), de função estrutural (*cf.* Wang & Wang, 2003) (Figura 4). Os segmentos S4 apresentam cargas elétricas positivas por terem em sua composição os aminoácidos arginina e lisina. Eles são denominados como "sensores de voltagem", porque são responsáveis pelo mecanismo de ativação e abertura do canal de Na^+ (Noda, 1993). Estudos com mapeamento estrutural dos canais de Na^+ sugerem que o "portão de ativação" do canal está localizado próximo da terminação citoplasmática do segmento S6 (*cf.* Wang & Wang, 2003). Recente trabalho indica que as toxinas ativadoras de canais de Na^+, como a veratrina, ligam-se ao poro interno da região do portão, modificando a

configuração deste e ocasionando a abertura do canal. Afirmam os autores que a ligação da veratrina ao canal leva à perda de seletividade permitindo a passagem de outros cátions, que não apenas o sódio (Tikhonov & Zhorov, 2005). Mutações em genes que codificam as proteínas que formam os canais de Na^+ são responsáveis pelo aparecimento de doenças chamadas "channelpathies". Cerca de vinte doenças de canais de sódio foram descritas e afetam a contração muscular, o batimento cardíaco e a função neuronal, acarretando condições incapacitantes para os indivíduos portadores destas doenças (*cf.* George, 2005).

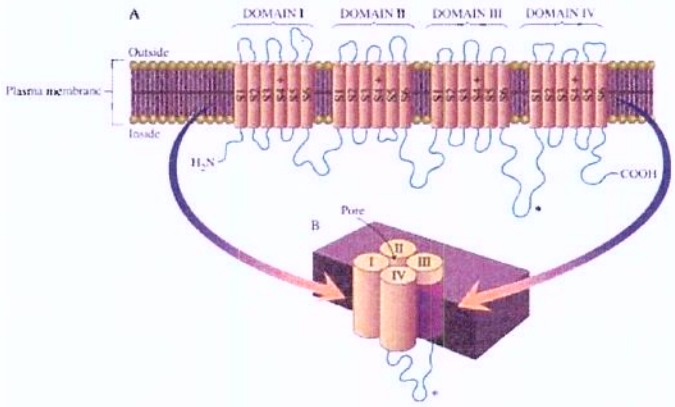

Figure 4: A – Esquema estrutural da subunidade α do canal de Na^+-dependente de voltagem; B – A formação do poro do canal (Fonte: http://www.ic.sunysb.edu)

O aumento da permeabilidade ao íon Na^+ e a ativação persistente dos canais de Na^+-dependentes de voltagem provocada pela veratrina pode ser inibido pela tetrodotoxina (TTX), um composto guanidínico heterocíclico que bloqueia esses canais iônicos (Catterall, 1980; Vital Brazil & Fontana, 1985).

1.1.3. Efeitos clínicos

O primeiro relato da utilização clínica dos extratos brutos de plantas do gênero *Veratrum* em pacientes com hipertensão arterial ocorreu em 1867 (Benforado, 1967; Catterall, 1980), por causa de sua ação hipotensora. Entretanto, Jarisch baseando-se nos estudos de von Bezold em 1939, observou efeitos cardiovasculares, como hipotensão e bradicardia, após a administração de pequenas doses intravenosas de veratrina em animais e humanos. (*cf.* Benforado, 1967). As ações cardiovasculares induzidas pela veratrina foram denominadas de "efeitos Bezold-Jarisch", termo sugerido por Krayer em 1953 (*cf.* Benforado, 1967).

Os alcalóides do *Veratrum* constituem um grupo de drogas antihipertensivas que atuam através de um mecanismo central indireto, de natureza reflexa, que foram inicialmente utilizados clinicamente. Nas décadas de 50 e 60, os alcalóides *Veratrum* foram empregados no tratamento de eclampsia, encefalopatia e falência ventricular esquerda relacionada com crises hipertensivas. Contudo, foram observados efeitos colaterais advindos destes alcalóides como paralisia da face e tórax, apnéia, miotonia, náusea e vômito, o que levou à restrição dos seus usos terapêuticos (Benforado, 1967).

1.1.4. Estudos *in vitro*

Diferentes métodos fisiológicos e farmacológicos têm sido empregados para o estudo de toxinas que atuam em canais de Na^+-dependentes de voltagem (Catterall, 1980). Através da técnica de "voltage-clamp", Ulbricht (1965; 1969) observou que o alcalóide veratridina causava despolarização nos nodos de Ranvier de fibras nervosas de rã e aumento na permeabilidade de Na^+, devido à ativação de canais de Na^+.

Em 1985, Vital Brazil & Fontana verificaram que a veratrina induz despolarização em regiões distintas em preparações frênico-hemidiafragma de ratos, dependendo da concentração utilizada do alcalóide no meio de incubação. Os autores concluíram que esta despolarização seletiva de regiões do músculo diafragma poderia ser causada por uma distribuição diferenciada de canais de Na^+ ativados ou pela existência de quantidades diferentes de tipos de canais de sódio em áreas distintas do músculo (Vital Brazil & Fontana, 1985). Posteriormente, os autores demonstraram que a veratrina e outras toxinas ativadoras dos canais de Na^+, tais como a miotoxina crotamina presente na composição do veneno da serpente *Crotalus durissus terrificus* (variedade *crotaminicus*), o veneno bruto da aranha *Phoneutria nigriventer* e sua fração tóxica PhTx2, produziam maior despolarização nas regiões do sarcolema próximas às junções neuromusculares (Vital Brazil & Fontana, 1993). Diferentemente destas toxinas, a veratridina e a batrachotoxina (BTX), ambos alcalóides, porém o último extraído da pele de rãs do gênero *Phyllobates*, despolarizavam 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 subtipos de canais de Na^+ encontrados nas regiões juncionais e extrajuncionais sensíveis de modo distinto às toxinas (Vital Brazil & Fontana, 1993).

Além da ativação dos canais de Na^+-dependentes da voltagem, os alcalóides do *Veratrum* podem alterar a fisiologia dos canais de Ca^{+2}, dependendo da concentração utilizada. Baixa concentração de veratridina (1 μM) não afeta as correntes de íons Na^+ e Ca^{2+} em fibras isoladas do músculo semitendinoso de rãs (Sutro, 1986). Entretanto, Nánási e colaboradores (1994) mostraram que a veratridina em altas concentrações (100 μM) ativa esses canais

iônicos. Os autores acreditam que o efeito da veratridina nos canais de Na^+ e Ca^{+2} seja explicado pela conhecida homologia existente na estrutura destes dois canais iônicos (Nánási *et al.*, 1994).

Têm sido observado também que a ação da veratridina sobre as concentrações intracelulares de cálcio e sódio, alteram conseqüentemente a respiração celular. Após incubação com diferentes concentrações de veratridina, estudos bioquímicos mostraram aumento do consumo de oxigênio e da captação de Ca^{2+} em cardiomiócitos (Moreno-Sánchez & Hansford, 1988) e em sinaptossomas isolados de ratos (Wermelskirchen *et al.*, 1992). Além disso, o alcalóide, causa ativação de caspases, geração de espécies reativas de oxigênio (ROS), fragmentação do DNA e indução de apoptose em células isoladas (Jordán *et al.*, 2000; 2002; 2003; Callaway *et al.*, 2001). Os autores sugerem que a veratridina age diretamente sobre os canais de Na^+-dependentes de voltagem e/ou sobre o trocador Na^+-Ca^{+2} (Moreno-Sánchez & Hansford, 1988; Wermelskirchen *et al.*, 1992), acarretando conseqüentemente um aumento no influxo de Ca^{2+}, o qual é responsável pelo desencadeamento da cascata de fatores pré-apoptóticos (Jordán *et al.*, 2000; Callaway *et al.*, 2001) e do estresse oxidativo (Chinopoulos *et al.*, 2000).

Também recentemente foi mostrado que a veratridina é capaz de aumentar a expressão da isoforma α_1 da Na^+-K^+-ATPase em cultura de células de músculo esquelético de galinha (Taormino & Fambrough, 1990) e de rato (Ladka & Ng, 2000). O tratamento com TTX causa declínio significante do número de moléculas da enzima (Brodie & Sampson, 1989) e reverte o aumento da expressão da mesma causado pela veratridina (Wolitzky *et al.*, 1986). A Na^+-K^+-ATPase é uma enzima constitutiva do sarcolema de fibras musculares esqueléticas, envolvida

no transporte ativo de Na^+ e K^+ e na manutenção do equilíbrio eletroquímico desses cátions (Harrison *et al.*, 1997; Nielsen & Clausen, 1996; Nielsen & Overgaard, 1996). Harrison *et al.* (1997) através de estudos com veratridina e aconitina, verificaram que a razão entre excitação induzida pelo influxo de sódio e a capacidade da bomba Na^+-K^+ é um fator determinante para a resistência do músculo e a taxa de retorno da força no músculo esquelético, indicando a dependência da performance contrátil e a funcionalidade da bomba. O maior "pool" de K^+ do organismo e a maior porcentagem de bomba Na^+/K^+ são encontrados no músculo esquelético (Clausen & Everts, 1989).

Atualmente, os alcalóides *Veratrum*, mais particularmente a veratrina e veratridina, têm sido muito utilizados em modelos experimentais de estudo de diversas doenças neurodegenerativas como Alzheimer (Kar *et al.*, 1998) e Parkinson (Loschmann *et al.*, 1995), e em casos de hipóxia (Banasiak *et al.*, 2004). A despolarização de membranas excitáveis causada por veratrina ou veratridina induz um aumento na liberação do aminoácido excitatório glutamato e nos níveis de guanosina monofosfato (GMP cíclico) (Reiser, 1990; Lizasoain et al., 1995; Shimojo *et al.*, 1999; Oka *et al.*, 2004), fatores envolvidos no desencadeamento de morte neuronal (Savolainen *et al.*, 1998).

1.1.5. Estudos *in vivo*

Pouco se sabe a respeito da ação tóxica dos alcalóides *Veratrum* em músculos esqueléticos utilizando-se modelos animais.

Nossos recentes trabalhos mostraram que a injeção intramuscular de 10 µg/ml de veratrina altera a atividade metabólica de diferentes fibras musculares em peixes e roedores

(Freitas *et al.*, 2001; 2002). Através do método histoquímico de NADH-tetrazólio redutase, reação que determina a atividade oxidativa das fibras musculares, observamos que as fibras respondem de formas distintas ao alcalóide dependendo do músculo e tempo de sobrevida analisado (Freitas *et al.*, 2001; 2002). Na musculatura lateral dos peixes tilápias (*Oreochromis niloticus*), as fibras musculares tipo I (oxidativas – ricas em mitocôndrias) tiveram um decréscimo da atividade de NADH-TR, enquanto as fibras tipo IIA e IIB (glicolíticas), exibiram aumento dessa atividade (Freitas *et al.*, 2001). Em contraposição, nos músculos sóleo e *extensor digitorum longus* (EDL) de camundongos, houve aumento na reação para NADH-TR nos três tipos de fibras musculares citados anteriormente (Freitas *et al.*, 2002). Além das alterações metabólicas, observamos que a veratrina afeta a atividade ATPásica miosínica de maneira distinta nos diferentes intervalos de tempo, músculos e tipos de fibras analisados (Freitas *et al.*, 2001; 2002).

A veratrina também provoca alterações ultraestruturais importantes nos músculos de peixes e mamíferos, tais como dilatação das cisternas terminais do retículo sarcoplasmático, desarranjo e rupturas das miofibrilas e variações no tamanho e morfologia das mitocôndrias (Freitas *et al.*, 2001; Cruz-Höfling *et al.*, 2002). Os autores concluíram que os efeitos tóxicos da veratrina induzidos nos músculos são resultantes do influxo excessivo de Na^+, seguido de fluido para o interior das fibras musculares, já que essas alterações foram antagonizadas pelo pré-tratamento com TTX (Freitas *et al.*, 2001; Cruz-Höfling *et al.*, 2002). Aventamos a possibilidade de que esses distúrbios poderiam induzir alterações no metabolismo do cálcio, por interferir no trocador Na^+/Ca^{+2}, já identificado no músculo esquelético (Balnave & Allen, 1998), onde o acúmulo de sódio intracelular reverte o mecanismo do trocador, fazendo-o trocar a entrada de um Ca^{2+} pela saída de três Na^+. Como resultado há aumento da

concentração de cálcio citossólico, também corroborado pelas respostas iterativas induzidas pela veratrina (Freitas *et al.*, 2001). Por outro lado, o acúmulo intracelular de sódio, desde que não compensado pelo efeito tamponante do retículo sarcoplasmático e das mitocôndrias, é deletério à célula, e pode contribuir para os danos causados às fibras musculares. Estes resultados despertaram o nosso interesse pelo estudo da ação do alcalóide veratrina, particularmente sobre as mitocôndrias de músculo esquelético e o retículo sarcoplasmático, constituindo estes, os focos desta tese.

1.2. Mitocôndrias

1.2.1. Estrutura

As mitocôndrias são normalmente descritas como estruturas cilíndricas e alongadas, com diâmetro de 0,5 a 1 μm e responsáveis pela respiração celular (*cf.* Alberts, 2002).

Graças aos avanços em técnicas de microscopia eletrônica, a estrutura das mitocôndrias foi desvendada. Palade e Sjöstrand foram os pioneiros a descrever a organização interna da mitocôndria. De acordo com os modelos propostos por estes autores, a mitocôndria era composta por sistemas de membranas, sendo que a membrana interna emitia septos (denominadas cristas) para o interior da matriz mitocondrial (Palade, 1952; Sjöstrand, 1956). Sabe-se hoje que a mitocôndria é limitada por dupla membrana fosfolipídica altamente especializada (a membrana externa e a membrana interna), definindo dois compartimentos, o espaço interno da matriz e o espaço intermembranas (*cf.* Alberts, 2002) (Figura 5).

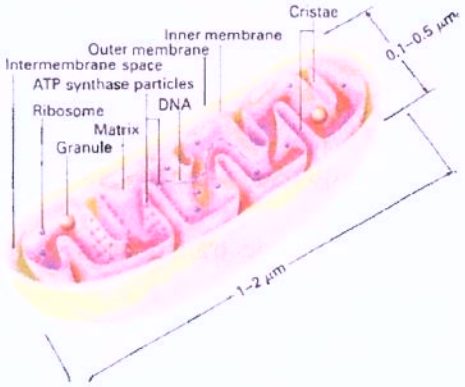

Figura 5: Esquema estrutural da mitocôndria (Fonte: http://www.elmhurst.edu)

A membrana externa da mitocôndria é permeável a íons e solutos com tamanho até 14 kDa, e aos substratos da cadeia respiratória (Wallace & Starkov, 2000). A bicamada lipídica é atravessada por proteínas transmembranas denominadas porinas, que formam poros na membrana e são ricas em colesterol. (*cf.* Van Gurp *et al.*, 2003). A membrana interna mitocondrial é seletivamente impermeável, exceto para certos íons e metabólitos, e é constituída principalmente pela cardiolipina (*cf.* Van Gurp *et al.*, 2003), um fosfolipídio específico desta membrana (Fleisher *et al.*, 1967). Nas membranas das cristas mitocondriais localizam-se os complexos de proteínas transportadoras de elétrons e a ATPase sintase (*cf.* Alberts, 2002). A matriz contém enzimas, principalmente as pertencentes ao ciclo de Krebs, ribossomos e DNA mitocondrial (*cf.* Alberts, 2002).

Em 1966, Hackenbrock aliou os ensaios bioquímicos com a análise morfológica de mitocôndrias isoladas de fígado de rato. O autor observou que dependendo do estado respiratório em que a mitocôndria se encontrava, ela adquiria uma configuração estrutural específica que era reversível. Assim, no estado 4 da respiração, ela apresentava configuração

ortodoxa, enquanto quando a mitocôndria estivesse no estado 3, ela apresentava a forma condensada (Hackenbrock, 1966).

Praticamente tudo que se conhece sobre a estrutura das mitocôndrias advém de estudos com a microscopia eletrônica de transmissão, porém alguns artefatos técnicos que existiam com a técnica foram reduzidos com o surgimento de técnicas de criofixação e criofratura. Através destas técnicas juntamente com recente recurso tecnológico da tomografia, houve uma melhor preservação da morfologia da organela, auxiliando assim o estudo da estrutura interna das mitocôndrias (Frey & Mannella, 2000). Reconstruções tomográficas da mitocôndria em 3D permitiram avaliar o formato e as dimensões dos sítios de contato entre as cristas e a membrana interna, e a interação destas organelas com outros componentes celulares, como por exemplo, a interação das membranas do retículo endoplasmático com a membrana mitocondrial (Frey & Mannella, 2000).

1.2.2. A Cadeia Respiratória

A descoberta em 1948 por Eugene Kenedy e Albert Lehninger de que as mitocôndrias eram os sítios da fosforilação oxidativa nos seres eucariotos, revolucionou os estudos de bioenergética (Wadkins & Lehninger, 1959; Lehninger, 2002).

Quatro complexos protéicos da cadeia respiratória (complexos I, II, III e IV), localizados nas cristas da membrana interna mitocondrial, mediam a transferência de elétrons do NADH ou $FADH_2$ para o aceptor final oxigênio e estes elétrons atravessam a bomba ATP sintase formando o ATP, processo denominado de fosforilação oxidativa (Figura 6). O transporte de elétrons é acoplado à geração de uma força próton-motriz na membrana interna. Esta força é representada pela combinação de um gradiente químico de prótons (pH), formado

a partir da ejeção de H^+ para o lado citossólico da membrana interna à medida que ocorre o transporte de elétrons, e o potencial elétrico de membrana ($\Delta\psi_m$), sendo esta a teoria quimiosmótica de conservação de energia proposta por Peter Mitchell em 1961. O refluxo de prótons para o interior da matriz é acoplado a síntese de ATP (ADP + fosfato inorgânico → ATP), através da bomba ATP sintase. Desta forma, a produção de ATP é dependente da integridade da membrana interna mitocondrial (*cf.* Van Gurp *et al.*, 2003).

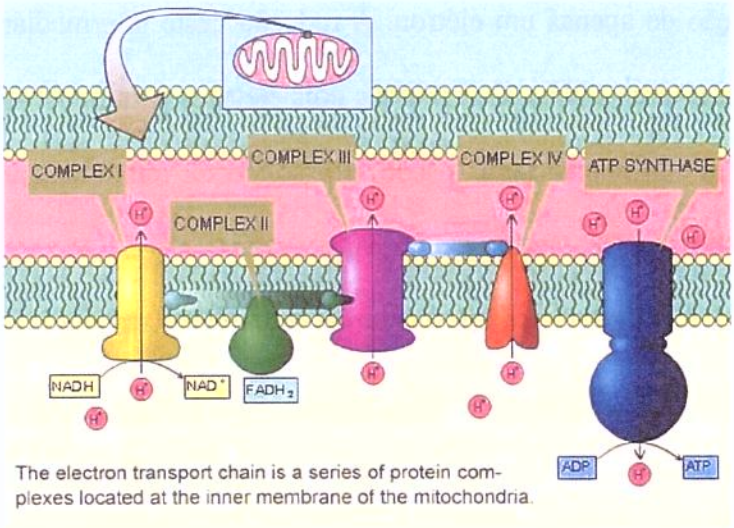

Figura 6: Modelo esquemático dos complexos protéicos da cadeia respiratória inseridos na membrana interna da mitocôndria (Fonte: http://www.ic.sunysb.edu)

Existem várias substâncias naturais ou sintéticas, que inibem ou desacoplam a respiração mitocondrial, por atuarem especificamente em um dos complexos protéicos.

1.2.2.1. Complexo I

A NADH-Q redutase ou NADH desidrogenase é o primeiro complexo de transporte de elétrons da cadeia respiratória mitocondrial. É um grande complexo estrutural enzimático, com cerca de 900 kDa consistindo de 34 cadeias polipeptídicas (*cf.* Stryer, 1996).

Este complexo oxida o NADH e transfere seus dois elétrons via os grupamentos prostéticos flavina mononucleotídeo (FMN) e os aglomerados ferro-enxofre para a ubiquinona (coenzima Q). A ubiquinona é reduzida a um radical livre anionte denominado semiquinona pela captação de apenas um elétron. A redução deste intermediário por um segundo elétron, origina o ubiquinol (QH_2). O fluxo dos dois elétrons para o QH_2 proporciona o bombeamento de quatro H^+ da matriz para o lado citossólico da membrana interna mitocondrial (Stryer, 1996; Lümmen, 1998; Dutton *et al.*, 1998).

Existem algumas toxinas naturais e também inseticidas e acaricidas sintéticos que agem no sítio da redução da ubiquinona, inibindo o complexo I da cadeia respiratória (Miyoshi *et al.*, 1998; Lümmen, 1998). As toxinas rotenona, isoflavonóide obtido das raízes da planta *Derris spec.* (Leguminosae), e o antibiótico piericidina A, isolado a partir da espécie *Streptomyces morabaraensis*, são os inibidores mais conhecidos e potentes do complexo I e amplamente utilizados em ensaios bioquímicos (Esposti, 1998; Lümmen, 1998).

1.2.2.2. Complexo II

Através do complexo succinato-Q redutase ou complexo II, o $FADH_2$ transfere seus elétrons para a ubiquinona, os quais se dirigem aos outros complexos da cadeia respiratória. Entretanto, este complexo não é uma bomba de prótons, diferentemente do complexo I, sendo assim forma-se menos ATP pela oxidação do $FADH_2$ do que pela do NADH (Stryer, 1996). A

estrutura química sintética 2-tenoiltrofluoroacetona é um exemplo de inibidor específico do complexo II (Luciani, 1971).

1.2.2.3. Complexo III

O complexo ubiquinol-citocromo c oxidoredutase ou complexo b-c_1 ou complexo III tem a função de transferir os elétrons do ubiquinol para o citocromo c. Nos mamíferos, o complexo III é constituído por 11 subunidades, incluindo o citocromo b e c_1 que contêm grupos prostéticos heme (Taylor *et al.*, 1994). A transferência de um par de elétrons por este complexo leva ao influxo de dois H^+ para o espaço intermembranas (*cf.* Stryer, 1996).

Dois importantes inibidores do complexo III são o mixotiazol e a antimicina A. O mixotiazol é produzido pela bactéria *Myxococcus fulvus* e age neste complexo, bloqueando a oxidação do ubiquinol (Wallace & Starkov, 2000). Já o antibiótico antimicina A, produzido a partir de várias espécies de fungos *Streptomyces*, inibe a transferência do elétron do grupo heme b para a molécula de quinona ou semiquinona (Wallace & Starkov, 2000).

1.2.2.4. Complexo IV

O citocromo-oxidase é o último complexo da cadeia respiratória e tem a função de transferir os elétrons do citocromo c para o aceptor final, o oxigênio. A redução do oxigênio em água, bombeia prótons da matriz para o lado citossólico da membrana interna da mitocôndria. O complexo é formado por 10 subunidades e contém dois grupamentos prostéticos heme A e dois iontes cobre (*cf.* Stryer, 1996).

O cianeto, a azida e o monóxido de carbono (CO) são exemplos clássicos de inibidores deste complexo. O cianeto e a azida ligam-se à forma férrica do citocromo a_3, de maneira não

competitiva com o oxigênio, enquanto o CO liga-se à forma ferrosa, competindo com o oxigênio (Wallace & Starkov, 2000).

1.2.2.5. ATP sintase

Em condições fisiológicas normais, os prótons que foram ejetados durante a transferência de elétrons na cadeia respiratória tendem a retornar para a matriz através da bomba ATP sintase ou complexo F0-F1. A ATP sintase utiliza a força próton-motriz para sintetizar ATP a partir do ADP e fosfato inorgânico, sendo que nos animais aeróbios é a principal fonte de ATP (Junge *et al.*, 1997).

O complexo F0-F1 possui duas estruturas protéicas, sendo F0 a porção transmembrana que permite o retorno de H^+ para o interior da matriz mitocondrial, e a porção F1 responsável pela síntese ou hidrólise do ATP (Junge *et al.*, 1997). A porção solúvel F1 contém três sítios catalíticos na subunidade central $\alpha_3\beta_3$ que se conecta a porção F0 através de duas subunidades b, formando então o complexo $\alpha_3\beta_3\delta ab_2$ (*cf.* Boyer, 2002; Kadenbach, 2003). Na porção F0, existem as subunidades c que funcionam como rotores impulsionados pela força próton-motriz ou pela demanda de ATP (*cf.* Kadenbach, 2003). Dependendo da espécie animal, a ATP sintase consiste de oito subunidades que possuem mais de 20 cadeias polipeptídicas, e representa cerca de 15% das proteínas totais da membrana interna mitocondrial (Junge *et al.*, 1997; Alberts, 2002).

Os inibidores clássicos da ATP sintase são o diciclohexilcarbodiimida (DCCD) e o antibiótico oligomicina, isolado da espécie *Streptomyces diastochromogenes*. O DCCD e a oligomicina ligam-se covalentemente à subunidade c de F0, bloqueando o fluxo de prótons e, conseqüentemente inibindo a atividade da ATP sintase (Soper & Pedersen, 1976).

1.2.3. Desacopladores da Fosforilação Oxidativa

Os desacopladores da respiração mitocondrial são compostos que diminuem a produção de ATP mitocondrial, isto é, o transporte de elétrons pela cadeira respiratória prossegue normalmente, porém a força próton-motriz é dissipada na presença destes compostos, não ocorrendo à síntese de ATP (*cf.* Stryer, 1996, Wallace & Starkov, 2000).

Existe uma variedade de substâncias químicas sintéticas, principalmente drogas e pesticidas que atuam como desacopladores. Os desacopladores mais comuns utilizados em ensaios bioquímicos são o 2,4-dinitrofenol (DNP), o carbonilcianeto-*m*-clorofenilhidrazona (CCCP) e o carbonilcianeto-*p*-trifluorometoxilfenilhidrazona (FCCP) (Kadenbach, 2003). Estes compostos são ácidos lipofílicos fracos que apresentam atividade protonoforética, isto é, atravessam a membrana interna na forma protonada, liberando o próton dentro da matriz mitocondrial (Wallace & Starkov, 2000).

Outros exemplos de desacopladores são os ionofóros, compostos capazes de transportar pequenos íons pela membrana mitocondrial, os ácidos graxos de cadeia longa (Wallace & Starkov, 2000; Kadenbach, 2003) e certos antiinflamatórios não-esteróides, tais como a nimesulida, o diclofenaco de sódio e o ácido mefênamico (Uyemura *et al.*, 1997; Caparroz-Assef *et al.*, 2001).

1.3. Retículo Sarcoplasmático

O retículo sarcoplasmático (RS), definição dada para caracterizar o retículo endoplasmático liso presente em fibras musculares, é uma rede de membranas ao redor de cada miofibrila (*cf.* MacLennan & Holland, 1976) (Figura 7). Possui dois compartimentos com funções distintas: cisternas longitudinais e as cisternas terminais.

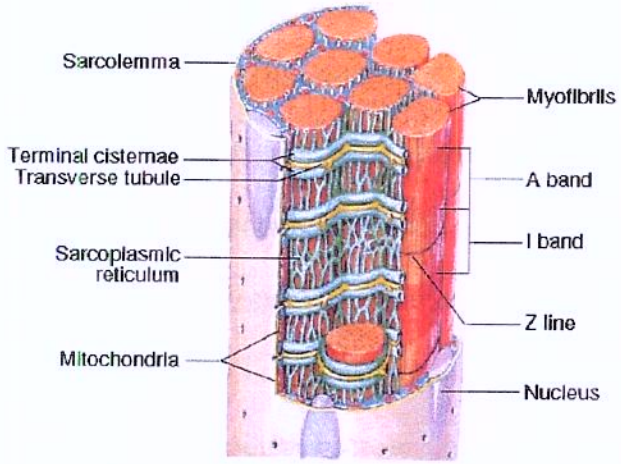

Figura 7: Modelo esquemático do retículo sarcoplasmático e do sistema de túbulos-transversos ao redor das miofibrilas (Fonte: http:// physioweb.med.uvm.edu/.../ cntrctl_excitation.htm)

As cisternas longitudinais do RS são responsáveis pela captação de íons cálcio do citoplasma através da bomba Ca^{+2}-ATPase, presente em suas membranas, deste modo interrompendo a atividade contrátil da fibra muscular (*cf.* MacLennan & Holland, 1976; Moller *et al.*, 2005). Já as cisternas terminais são responsáveis pelo armazenamento e liberação de Ca^{+2} do interior do RS após um estímulo químico ou elétrico. A liberação do Ca^{+2} é através de receptores rianodina presentes nestas cisternas, e a proteína calsequestrina liga-se aos íons cálcio no interior do retículo (Endo, 1977; Dulhunty *et al.*, 1996). A triadina é uma

proteína transmembrana presente também nas cisternas terminais do RS que interage com a calsequestrina ao mesmo tempo em que esta última liga-se ao receptor rianodina (Kim *et al.*, 1990). Existem várias isoformas do receptor rianodina no reino animal, que são modulados pelas concentrações de ATP, Mg^{2+} e Ca^{+2} no citoplasma, e pela concentração de Ca^{+2} no interior do retículo sarcoplasmático (Laver, 2005).

A estreita relação estrutural entre o RS e os túbulos transversos (túbulos-T) em vertebrados é estabelecida na região das cisternas terminais do RS, as quais ladeiam o túbulo-T, em estreita aposição, caracterizando a estrutura denominada "tríade", ou mais recentemente CRU ("calcium release unit") (Flucher & Franzini-Armstrong, 1996). As tríades se dispõem em registro e regularmente, sempre posicionadas no limite entre as bandas A e I, isto é, no local da intersecção dos miofilamentos de actina e miosina. Desta forma, o número de tríades por fibra muscular é o dobro do número de sarcômeros presentes nessa fibra. A localização topográfica da tríade na fibra é estratégica para os fenômenos de acoplamento excitação-contração (Flucher, 1992). A distância entre a membrana das cisternas terminais do RS e o túbulo-T é de 12 a 14 nm (Franzini-Armstrong, 1971). O potencial de ação é propagado da membrana sarcolemal para o sistema de túbulos-T, que interage com a membrana do RS na região das tríades. Assim, o potencial de ação é conduzido para o interior da fibra, promovendo o mecanismo de excitação-contração muscular (Eisenberg & Eisenberg, 1982).

1.4. Ação de toxinas e venenos ativadores de canais de Na^+ em mitocôndrias e no retículo sarcoplasmático isolados

Existe uma variedade de toxinas e venenos naturais que interferem na bioenergética mitocondrial e na fisiologia do retículo sarcoplasmático.

Estudos têm mostrado que os venenos brutos de serpentes e suas frações tóxicas induzem alterações estruturais e funcionais em mitocôndrias isoladas como entumescimento, inibição e/ou descoplamento da respiração (Condrea *et al.*, 1965; Valente *et al.*, 1998). As fosfolipases presentes nestes venenos atuam sobre a membrana mitocondrial, liberando ácidos graxos que ocasionam permeabilidade da membrana e alteração na oxidação fosforilativa (Condrea *et al.*, 1965; Elliot *et al.*, 1966; Nicholls *et al*, 1985; Valente *et al.*, 1998).

Da mesma forma que o alcalóide veratrina, certas toxinas dos venenos de espécies de escorpiões (β-toxinas escorpiônicas) ligam-se a sítios específicos nos canais de Na^+-dependentes de voltagem, modificando a fisiologia destes canais (Zuo & Ji, 2004). Além deste mecanismo, eles podem também alterar o metabolismo das mitocôndrias. Toxinas isoladas do veneno bruto do escorpião *Buthus martensii Kashi*, por exemplo, inibem a oxidação fosforilativa, diminuem a atividade da citocromo oxidase e aumentam a fluidez da membrana mitocondrial (Song & Yang, 1986). Já o veneno bruto do escorpião *Heterometrus fulvipes* causa o desacoplamento da respiração, diminuição das atividades do succinato e glutamato desidrogenases e inchaço mitocondrial (Venkaiah & Parthasarathy, 1983).

A miotoxina *a* é um exemplo de polipeptídico básico que ativa também os canais de Na^+-dependentes de voltagem. Esta toxina é isolada da peçonha da cascavel norte-americana *Crotalus viridis viridis* (Cameron & Tu, 1978) e causa primeiramente vacuolização das cisternas do retículo sarcoplasmático, e posteriormente, desorganização das miofibrilas

(Ownby *et al.*, 1976). A batrachotoxina (BTX) também leva ao aparecimento de vacúolos na fibra muscular, vacúolos esses decorrentes do aumento em volume das cisternas terminais do RS (Albuquerque *et al.*, 1971; Warnick *et al.*, 1971). A BTX se caracteriza por ativar canais de sódio-dependentes de voltagem. Em 1986, Volpe e colaboradores demonstraram que a miotoxina *a* provoca o desacoplamento da bomba Ca^{+2}-ATPase em vesículas isoladas de retículo sarcoplasmático, inibindo a recaptação de íons cálcio (Yudkowsky *et al.*, 1994). O aumento da concentração intracelular de Ca^{2+} causado pela miotoxina poderia ser diretamente responsável pelas alterações morfológicas observadas nas fibras musculares por outros autores (Ownby *et al.*, 1976; 1982; Cameron & Tu, 1978). A hipótese proposta por Ownby *et al.* (1976) para explicar o aparecimento de vacúolos surgidos do aumento das cisternas terminais do RS após injeção i.m. da miotoxina *a*, do veneno de *C. v. viridis*, foi de que a interferência no transporte de Na^+ pela toxina se daria pela inibição da Na^+-K^+ATPase presente no sarcolema. O aumento do influxo de Na^+ levaria ao aumento do influxo de água, ao edema celular e por fim à morte da célula. Ownby *et al.* (1976) não analisaram se as alterações envolvendo o retículo sarcoplasmático poderiam ser revertidas pelo tratamento com a TTX, bloqueadora dos canais de sódio, e portanto, impedindo o aumento da permeabilidade ao Na^+. Chang & Tseng (1978), relataram que a crotamina, toxina do veneno da cascavel sul-americana *C. durissus terrificus*, induziu o influxo de Na^+, e que o pré-tratamento com ouabaína, um inibidor da Na^+-K^+ATPase, potenciava os efeitos da crotamina. A conclusão dos autores foi de que a crotamina se ligava a uma molécula que restringiria a permeabilidade do canal de sódio aos íons Na^+, causando por isso permeabilidade aumentada a esses íons. Esta permeabilidade aos íons sódio induzido pela crotamina foi prevenida pela TTX (Pellegrini *et al.*, 1978; Chang & Tseng, 1978). Também, a diminuição da concentração de Na^+ extracelular,

no meio de incubação de preparações neuromusculares *in vitro*, preveniam os efeitos da crotamina (Pellegrini *et al.*, 1978; Chang & Tseng, 1978). A crotamina injetada em músculo esquelético de camundongos induz a formação de inúmeros vacúolos na fibra muscular, entretanto não se pode afirmar se esses vacúolos tenham origem nas cisternas terminais do retículo sarcoplasmático, uma vez que essas observações foram feitas com microscopia de luz (L.S.Queiroz, comunicação pessoal).

1.5. Comunicação cruzada entre mitocôndrias e retículo sarcoplasmático no músculo esquelético

As mitocôndrias constituem uma das principais estruturas subcelulares no músculo esquelético. O papel-chave destas organelas na fisiologia muscular esteve sempre associado à produção de energia através da geração de ATP. Além desta função, que está bem estabelecida e documentada, há evidências acumuladas mais recentes, relativas à importância do transporte do Ca^{+2} na homeostase intracelular de Ca^{+2} (Sembrowich *et al.*, 1985; Madsen *et al.*, 1996; Gillis, 1997; Isaeva & Shirokova, 2003; Isaeva *et al.*, 2005). Há confirmação de que a captação de Ca^{+2} pela mitocôndria desempenha um papel importante na regulação dos sinais de Ca^{+2} durante o ciclo de contração-relaxamento no músculo esquelético de mamíferos (Bruton *et al.*, 2003; Lannergren *et al.*, 2001). Até momento, o dado mais marcante sobre a participação mitocondrial no acomplamento excitação-contração do músculo esquelético foi apresentado por Rudolf *et al.* (2004). Os autores relataram um rápido aumento na $[Ca^{2+}]_m$ durante contrações isoladas ou estimulação tetânica em camundongos *in vivo*. Por outro lado, Shkryl & Shirokova (2006) demonstraram a proximidade funcional entre os sítios de liberação de Ca^{2+} do RS com os sítios de captação de Ca^{2+} mitocondrial em músculos de contração

rápida e lenta de ratos comparando as transições de Ca^{2+} citossólico e mitocondrial induzidas pela cafeína na presença de tampões de Ca^{2+} lento (EGTA) e rápido (BAPTA). Os autores mostram evidências da existência de microdomínios funcionais, e provavelmente estruturais, altamente localizados durante a liberação de Ca^{2+}, domínios esses que incluiriam tanto os sítios de liberação de Ca^{2+} do RS, como os sítios de captação de Ca^{2+} mitocondrial, e que existem subpopulações de mitocôndrias posicionadas estrategicamente de acordo com requisitos funcionais. A estreita proximidade estrutural existente entre retículo endoplasmático (retículo sarcoplasmático) e mitocôndrias, sugestivas da existência de conversas cruzadas entre ambas as organelas, tem sido documentada em diferentes tipos de células e também no músculo cardíaco e esquelético. No presente trabalho, focamos a ação da veratrina sobre componentes da fibra muscular esquelética, particularmente em seus efeitos sobre as mitocôndrias e retículo sarcoplasmático.

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3. OBJETIVOS GERAIS

Dando continuidade aos nossos estudos sobre os efeitos tóxicos da veratrina no músculo esquelético realizados durante o mestrado, tivemos como objetivos nesta etapa:

- Avaliar o potencial tóxico do alcalóide sobre mitocôndrias isoladas de músculos esqueléticos e comparar com efeitos causados *in vivo* nos músculos sóleo e EDL de camundongos.
- Avaliar por meio de métodos citoquímicos se a veratrina afeta a atividade das enzimas mitocondriais nicotinamida adenina dinucleotídeo desidrogenase (NADH desidrogenase), succinato desidrogenase (SDH) e citocromo oxidase (COX).
- Investigar se os alcalóides veratrina e veratridina afetam a bioenergética de mitocôndrias isoladas de músculo e fígado.
- Avaliar a ação da veratrina sobre a ultraestrutura do retículo sarcoplasmático, comparando quatro tipos de músculos esqueléticos: diafragma, EDL, sóleo de camundongos, e músculo lateral de peixes.

4. RESULTADOS

Os dados resultantes desta tese de doutorado originaram três artigos científicos. O primeiro artigo intitulado "Effects of veratrine on skeletal muscle mitochondria:ultrastructural, cytochemical and morphometrical studies" enfoca os efeitos tóxicos da veratrina sobre o consumo de oxigênio e a atividade das enzimas da cadeia respiratória de mitocôndrias isoladas de músculos esqueléticos de ratos (NADH desidrogenase, succinato desidrogenase e citocromo oxidase), através de ensaios bioquímicos e citoquímicos. Além disso, comparamos as alterações ultraestruturais provocadas pela mistura de alcalóides nas mitocôndrias de músculos esqueléticos tanto nos estudos *in vivo* quanto nos *in vitro*. Este trabalho foi publicado na revista científica internacional *Microscopy Research & Technique* (69: 108-118, 2006), da editora Wiley-Liss, EUA, e com fator de impacto de 2,609.

O segundo artigo científico com o título de "Effects of veratrine and veratridine on oxygen consumption and electrical membrane potential of isolated rat skeletal muscle and liver mitochondria" refere-se ao comprometimento da função mitocondrial como consumo de oxigênio e potencial elétrico de membrana, após incubação de concentrações crescentes de veratrina. Entretanto, apenas as mitocôndrias isoladas de músculos esqueléticos de ratos mostraram-se sensíveis à toxicidade da veratrina, diferentemente das mitocôndrias hepáticas. O artigo também aborda a ação do alcalóide veratridina, presente na composição da mistura veratrina, nos parâmetros respiratórios de mitocôndrias isoladas. Este trabalho foi aceito para publicação na revista científica *Toxicon*, da editora Elsevier, Reino Unido, e com fator de impacto de 1,862.

O terceiro trabalho intitulado "Mitochondria and sarcoplasmic reticulum as cellular targets for veratrine" enfoca as alterações ultraestruturais induzidas pela veratrina em diferentes concentrações e tempos de observação em quatro músculos distintos: o músculo diafragma, EDL e sóleo de camundongos, e a musculatura lateral de peixes tilápias. Os resultados mostraram que as principais organelas danificadas pela mistura de alcalóides foram às mitocôndrias e o retículo sarcoplasmático. Neste estudo, sugerimos que a veratrina causou um distúrbio osmótico nas fibras musculares, alterando a permeabilidade das membranas das mitocôndrias e das cisternas terminais do retículo sarcoplasmático. Este último trabalho foi submetido à publicação na revista *Cell Structure and Function*, da *Japan Society for Cell Biology* e com fator de impacto de 2,932.

ARTIGO 1: EFFECTS OF VERATRINE ON SKELETAL MUSCLE MITOCHONDRIA: ULTRASTRUCTURAL, CYTOCHEMICAL AND MORPHOMETRICAL STUDIES

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EFFECTS OF VERATRINE ON SKELETAL MUSCLE MITOCHONDRIA: ULTRASTRUCTURAL, CYTOCHEMICAL AND MORPHOMETRICAL STUDIES

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Running title: Veratrine toxicity in isolated mitochondria

ABSTRACT

The alkaloid veratrine is a lipid soluble neurotoxin which target voltage-gated Na^+ channels for their primary action. Recently, we showed that this alkaloid may cause myonecrosis and evidences suggest mitochondria as one of its cell target. Herein we investigate the effects caused by variable concentration of veratrine (250-550 μg/ml) on mitochondrial oxygen consumption, respiratory chain enzymes activities and ultrastructure, combining electron microscopy with cytochemical and biochemical approaches. The results showed different sort of ultrastructural changes, both in isolated and intramuscular mitochondria. Veratrine decreased mitochondrial nicotinamide adenine dinucleotide dehydrogenase (NADH-d), succinic dehydrogenase (SDH) and cytochrome oxidase (COX) activities, inhibited significant and dose-dependently the state 3 respiration rate, respiratory control ratio (RCR) and ADP/O on isolated rat skeletal muscle mitochondria, whereas state 4 was unaffected. A tendency of increase in mitochondria diameter was seen with 250 μg/ml veratrine. We conclude that the alkaloid would probably act on mitochondrial membrane phospholipid configuration which would explain the changes observed.

Key words: Cytochemistry, mitochondria, morphometry, skeletal muscle, veratrine, ultrastructure

INTRODUCTION

Voltage-gated Na^+ channels used to be primary targets of a variety of naturally toxins and therapeutic drugs. Veratrine alkaloid, extracted from the seeds of some *Veratrum* plants of the Liliaceae family (Ulbricht, 1998), acts as activator of Na^+ channels on excitable membranes (Catterall, 1980) causing iterative depolarization (Vital Brazil and Fontana, 1985), higher influx of Na^+, which osmotically is followed by accumulation of fluid inside differently-sized newly-formed peri-, intra-axonal or intra-myelin vacuoles in nerve fibers (Cruz-Höfling and Vital Brazil, 1990).

Some studies utilized the veratrine as tool to induce increase of intracellular Na^+ and neuronal death, processes which are associated in many neurodegenerative disorders (Shimojo et al., 1999; Chinopoulos et al., 2000; Koike et al., 2000).

Recent experiments have suggested that the alkaloid veratrine may be involved in degenerative mitochondrial changes observed when the drug is injected intramuscularly (i.m.) in mice (Freitas et al., 2002; Cruz-Höfling et al., 2002). In muscle cells profound changes may also affect myofibrils and myofilaments organization, metabolic alterations and contractile fiber type transition, the extent and quality of which depended on the muscle type and animal species (Freitas et al., 2001; 2002; Cruz-Höfling et al., 2002). Such findings led us to suppose that the alkaloid may get access into the fibers, a view already raised by Honerjäger et al. (1992), probably facilitated by the alkaloid lipid-soluble biochemical nature (Catterall, 1980), and cell membrane bilayer properties. In this work, based on the differences of *extensor digitorum longus* (EDL) and *soleus* (SOL) muscles in their response to veratrine, seen *in vivo* (Freitas et al., 2002; Cruz-Höfling et al., 2002), we analyzed mice skeletal muscle

mitochondria by transmission electron microscopy (TEM). In addition, we determined oxygen consumption and analyzed the respiratory chain enzymes in isolated skeletal muscle mitochondria from rats, combining biochemical, cytochemical and morphometrical methods to confirm them as targets of veratrine.

MATERIAL AND METHODS

Animals

Adult Balb/c mice (20-25 g) and Wistar rats (200-250 g) were obtained from the university's Animal House Unit (CEMIB/UNICAMP – Campinas/SP). The animals 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*. All the procedures were in accordance with the guidelines of Brazilian College on Animal Experimentation (COBEA).

Chemicals

Veratrine hydrocloride was dissolved in distilled water adjusting pH 7.4 with KOH immediately before use. Veratrine, paraformaldehyde, osmium tetroxide, α-ketoglutarate, malate, glutamate, pyruvate, cytochromo c, catalase, nicotinamide adenine dinucleotide reduced (NADH), ADP, EGTA [ethylene glycol-bis(β-aminoethyl ether)N, N, N', N'-tetraacetic acid)], EDTA (ethylene diamine tetra acetic acid), BSA (bovine serum albumin) and Tris [Tris(hydroxymethyl)aminomethane] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glutaraldehyde was purchased from Polysciences, Inc. (Warrington, PA, USA). All other reagents were commercial products of the highest available grade of purity.

Ultrastructural studies of mice muscles (*in vivo*)

Balb/c mice were anesthetized with sodium pentobarbital (40-70 mg/kg) and submitted to a microsurgery that exposed the EDL and SOL muscles for intramuscular (i.m.) injection of

5 µl veratrine hydrocloride (10 µg/ml). After 15, 30 or 60 min of injection (n = 3/groups), mice under deep anesthesia were perfused through cardiac puncture into the left ventricle with Karnovsky fixative (2.5 % glutaraldehyde plus 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Control group received 0.85% sterile saline solution injection at the same experimental condition. The muscles were removed and sectioned into small fragments. Muscle fragments were immersed in Karnovsky solution for 3 h, post-fixed in 1% OsO_4 for one hour, dehydrated through graded acetone and embedded in an Epon-Araldite resin mixture. Sections (0.5-1 µm thick) were stained with toluidine blue and appropriate regions were selected by light microscopy for ultramicrotomy. Ultrathin sections (60-70 nm thick) were stained with lead citrate and uranyl acetate, and examined in transmission electron microscope (Leo 906 Zeiss EM), operated at 60 kV.

Isolation of rat skeletal muscle mitochondria

The Wistar rats were sacrificed through cervical dislocation before hindlimbs muscles were removed for the isolation of mitochondria by method of Tonkonogi and Sahlin (1997) modified. The muscles were dissected, perforated and immersed in ice-cold isolation buffer with the following composition: 100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, pH 7.4, 1 mM K_2HPO_4, 0.1 mM EGTA and 0.2 % BSA, at 4°C. After, the muscles were washed in ice-cold medium, finely minced with scissors and homogenized with a Polytron and Potter-Elvehjem-type homogenizer. The homogenate was centrifuged (4°C) at 700 Xg for 10 min. The supernatant was retrieved and centrifuged at 10000 g for 10 min. The resulting pellet was then resuspended in the same buffer and recentrifuged at 6000 Xg for 3 min.

The final mitochondrial pellet was resuspended in the medium consisting of 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl buffer, pH 7.4, 0.1 mM EDTA to a protein concentration of 30-40 mg/ml and kept on ice during all biochemical assays. Protein concentration was determined by the biuret method using BSA as standard (Gornall et al., 1949).

Biochemical assays

Oxygen consumption was measured with a Clark-type electrode (Yellow Springs Instruments Co., OH, USA) in 1.3 ml of standard reaction medium (28°C), in a sealed glass cuvette equipped with a magnetic stirrer. The reaction medium (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, pH 7.4, 10 mM K_2HPO_4, 0.1 mM EDTA) was added to 0.3 mg of mitochondrial protein from rat skeletal muscles, Complex I substrates cocktail (5 mM α-ketoglutarate, 5 mM malate, 5 mM glutamate and 5 mM pyruvate), 200 nmols of ADP and 250-550 µg/ml of veratrine hydrocloride. Control assays were done without the addition of veratrine. Each experiment was perfomed in triplicate. Respiratory rates and respiratory control ratio (RCR = state 3/state 4) were calculated according to Chance and Williams (1956).

Cytochemical studies

After biochemical assays with 250 and 500 µg/ml the mitochondrial pellets from rat skeletal muscles were collected and processed for cytochemical demonstration of NADH-d

(Hanker et al., 1973), SDH (Kerpel-Fronius and Hajós, 1968) and COX (Seligman et al., 1968) activities (n = 6/per assay).

Control experiments (n = 6) were performed with addition of specific inhibitors of the respiratory chain enzymes on incubation medium (0.05 M sodium malonate for SDH and 0.01 M potassium cyanide for COX) or omission of substrate on medium (NADH-d negative control).

Later, the mitochondrial pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 containing 2% sucrose for 30 min at 4°C. The pellets were resuspended in ice-cold phosphate buffer with 7.5% sucrose and centrifuged at 700 Xg for 5 min. Subsequently, the pellets were post-fixed in buffered 1% OsO_4, dehydrated through a graded series of acetone and processed for TEM. Ultrathin sections (60-70 nm thick), not contrasted with uranyl acetate and lead citrate, were examined in transmission electron microscope (Leo 906 Zeiss EM), operated at 60 kV.

Morphometry

The extent of damage caused by incubation of isolated rat skeletal muscle mitochondria with veratrine (250 and 500 µg/ml) was evaluated by measuring the diameter of mitochondria profiles taken from selected electron micrographs (NADH-d, SDH and COX reaction samples) all of which with X 6000 magnification. The images were analyzed on KS 300 program version 2.01 (Kontron Elektronik, Germany). The measurement of mitochondrial diameters was done using tpsDig program version 1.40 and expressed in percentage of pixel units (Rohlf and Marcus, 1993). In each experimental group (n = 6/treatment), five non-

overlapping fields of each ultrathin section obtained from control and veratrine-treated mitochondrial pellets were analysed. Only those mitochondria which were intact and exhibited sharp limiting membranes were selected for measurements. About two thousand of mitochondria were measured per treatment.

Statistical analysis

Respiratory rates and percentage of diameters from isolated rat skeletal muscle mitochondria were reported as mean values ± standard error. Analyses were done by one-way ANOVA followed by Bonferroni's *post hoc* comparisons. A probability of $P < 0.05$ was considered significant.

RESULTS

Mitochondria ultrastructural (*in vivo*) studies

TEM analysis of mice EDL muscle injected (i.m.) with saline solution showed normal-looking and normal-sized mitochondria. They were aligned within I bands of myofibrils, laterally to Z-lines, having side-by-side the triads. Both the cristae and matrix electron density were regular (**Fig. 1A**). As for EDL, the mitochondria from saline-treated SOL muscle were structurally normal, being found both in the intermyofibrillar and subsarcolemmal space (**Fig. 1B**).

Veratrine induced remarkable structural changes on the mitochondria as a whole, and in the shape and size of cristae. No apparent difference in the quality of changes was seen between mitochondria from EDL and SOL (Fig. **1C-H**). However, although the ultrastructural changes were already present soon after 15 min after veratrine injection in both muscles (**Fig. 1C-D**), degeneration was more widespread in SOL (**Fig. 1D**). The alterations started with an initial moderate increase of the organelle size and peaked at 30 min post-injection (**Fig. 1E-F**). The changes of mitochondria volume were short-lived, although some swelling still persisted 60 min after veratrine injection, particularly in the population of subsarcolemmal regions (**Fig. 1G-H**). Besides, changes involving the matrix and cristae were observed, as described in detail in the legend of figures (**Fig. 1C-H**). No indication of calcium accumulation was seen inside the mitochondria of EDL or SOL.

Inhibition of oxygen consumption of isolated rat skeletal muscle mitochondria by veratrine

There was inhibition of oxygen consumption by veratrine, the rate of which depended on the toxin concentration. Table 1 shows a significant decrease of state 3 respiration rate and RCR with 450 µg/ml and 550 µg/ml veratrine, respectively. The ADP/O ratio progressively decreased with increasing veratrine concentration. The state 4 respiration rate was not affected significantly by the alkaloid when compared to control (without addition of veratrine) (Table 1).

Effects of veratrine on morphology and respiratory enzymes activity of isolated skeletal muscle mitochondria

Ultrastructural analysis of controls isolated rat skeletal muscle mitochondria (veratrine-untreated) revealed normality in their round or oval shape, size, cristae pattern and matrix electron density (**Figs. 2A-B, 3A-B** and **4A-B**). However, some mitochondria were swollen and ruptured due to the isolation procedure.

On the other hand, mitochondria incubated with veratrine (250 µg/ml and 500 µg/ml) exhibited ultrastructural alterations that included irregular outline and/or rupture of the outer mitochondrial membrane, disarranged crista and matrix electronlucency (**Figs. 2C-G, 3C-G** and **4C-G**).

NADH-d and SDH: The cytochemical method for detecting NADH-d and SDH activities uses potassium ferricyanide, in which the ferricyanide anion acts as an acceptor of electrons from the mitochondrial respiratory chain, whose final reaction product is an insoluble precipitate (copper ferricyanide). The precipitate localization indicates the sites

where mitochondrial NADH-d and SDH activities are detectable (sites of Hatchett´s Brown) (Hanker et al., 1973).

In controls, the ultrastructural cytochemical demonstration of the NADH dehydrogenase activity is illustrated in **Figure 2A** and **B,** showing the electrondense reaction product impregnating the outer and inner membranes, as well as the membrane of the cristae of the mitochondria. At a concentration of 250 µg/ml veratrine, a tenuous (**Fig. 2C**) or none precipitate (**Fig. 2D**) was seen over the membranes. In addition distorted organelle outline was a common finding. With 500 µg/ml veratrine concentration, the response was not uniform, since morphologically unchanged, or abnormally-looking mitochondria, with positive or negative enzyme activity could be seen (**Fig. 2E-F**). The omission of substrate for NADH dehydrogenase acted as a negative control to the cytochemical reaction (**Fig. 2G**).

For the SDH reaction, both the mitochondrial membranes as the intracristae matrix showed positive labeling in veratrine-untreated controls mitochondria demonstrated through a thin electrondense deposit, when observed by TEM (**Figs. 3A-B**). However, after incubation with veratrine, besides abnormal morphology, mitochondria were in general negative for SDH activity (**Figs. 3C-F**). Moreover, veratrine-treated mitochondria presented electronlucent matrix devoid of cristae, as if they were "ghost organelles" (**Figs. 3C-F**). The reaction for SDH was found completely abolished when the mitochondria were incubated in a substrate medium containing sodium malonate (**Fig. 3G**).

The COX activity is cytochemically demonstrated by the oxidative polymerization of 3,3'-diaminobenzidine (DAB) resulting in an osmiophilic and amorphous product (Seligman et al., 1968). In control preparations, this reaction product indicating COX activity was found within cristae matrix and impregnating the outer and inner mitochondrial membrane (**Fig. 4A-**

B), whereas the majority of veratrine-treated mitochondria exhibited irregular patches of reaction (**Fig. 4C-F**), or showed a complete lack of reaction (**Fig. 4C, E-F**). The COX activity was inhibited by pre-treating the mitochondria pellets with KCN, used as control negative of the reaction (**Fig. 4G**).

Morphometric analysis

All the population of isolated rat skeletal muscle mitochondria incubated or not with veratrine exhibited a wide variability of diameter. A parcel of the mitochondria population exhibited small to median diameters, while others were swollen, and hence presented higher diameters. Therefore, there were no significant difference between control and veratrine-treated mitochondria in relation to their diameters, although a tendency of increase was observed in the veratrine-treated population, principally after incubation with 250 μg/ml veratrine concentration (**Fig. 5**).

DISCUSSION

The data presented in this report demonstrated that the administration of veratrine *in situ* and *in vitro* produces a variety of biochemical (decrease of oxygen consumption), cytochemical (total or partial inhibition of NADH-d, SDH and COX activities) and morphological changes (diameter alterations and cristae disorganization and/or rupture) in rodent skeletal muscle mitochondria. In relation to mitochondrial alterations, the mouse SOL muscle showed to be more sensitive to the myotoxic effects of veratrine than the EDL, confirming a previous report by Cruz-Höfling et al. (2002). That different muscle types have different sensitivities to toxins and animal venoms has already been reported (Harris et al., 1975; Harris and Johnson, 1978; Melo and Ownby, 1996; Freitas et al., 2001; 2002), indicating that intrinsic physiological and metabolic muscle properties may influence the extent of damage caused by these myotoxic agents. Recently, we have shown that veratrine causes necrosis in mice and fish muscle fibers characterized by hypercontraction of myofibrills, disorganization of sarcomeres, swelling of sarcoplasmic reticulum cisternae and mitochondrial damage, and that these alterations were prevented with tetrodotoxin pretreatment, a Na^+ channel-blocker (Freitas et al., 2001; Cruz-Höfling et al., 2002). At the time, we inferred that the majority of the *in vivo* alterations could have its basis on enhanced influx of Na^+ and water into cells and osmotic unbalance (Freitas et al., 2001; Cruz-Höfling et al., 2002). We also raised the possibility that mitochondria could be a fiber target for veratrine.

Veratrine is a lipid-soluble toxin that binds the receptor site type 2 of voltage-gated Na^+ channels, which it is localized on S6 transmembrane segment of Na^+ channel α-subunits (Wang and Wang, 2003). Although the clinical use of the crude extracts of the *Veratrum*

plants were employed therapeutically in hypertensive patients since the late 18th century, a series of harmful collateral cardiovascular symptoms, generically named "Bezold-Jarisch effects", restricted the use of this alkaloid in clinical trials (Benforado, 1967). Currently, this alkaloid has been more utilized as a pharmacological tool to understand the mechanism of Na^+ channel gating (Felix et al., 2004; Son et al., 2004; Weiser, 2004). Depolarization of nerve fibers induced by veratridine, an alkaloid present in veratrine (Catterall, 1980), increases the release of excitatory amino acid glutamate, nitric oxide (Lizasoain et al., 1995) and production of reactive oxygen species (Chinopoulos et al., 2000; Jórdan et al., 2002; 2003). Since oxidative stress and glutamate-induced neurotoxicity may be involved in neuronal death by apoptosis observed in various neurological disorders, such as epilepsy, Alzheimer's disease and other brain injuries (Savolainen et al., 1998), the drug has been also used as experimental model to mimic pathophysiological alterations seen in Alzheimer's (Kar et al., 1998) and Parkinson's diseases (Loschmann et al., 1995).

Oxidative stress generated by veratridine (Chinopoulos et al., 2000; Jórdan et al., 2000; 2002; 2003) may be a sign that the *Veratrum* alkaloids act directly on mitochondrial respiration. Different patterns of mitochondrial abnormalities, as swelling and disruption of cristae seen in our experiments *in vivo*, have been usually described on reports of neuromuscular disorders (Lindal et al., 1992; Nishino et al., 1998; Santoro et al., 2004), and in studies using oxidative phosphorylation uncouplers (Melmed et al., 1975; Sahgal et al., 1979; Shah et al., 1982). There are evidences that disturbance on intracellular concentration of calcium ions may play a significant role on mitochondria alterations induced by natural toxins (Gopalakrishnakone et al., 1984; Gutiérrez et al., 1984; 1991; Cruz-Höfling and Rodrigues-Simioni, 1998; Mattiello-Sverzuta and Cruz-Höfling, 2000). Studies have shown that

veratridine not only alters the Na^+ influx but also modifies the properties of Ca^{2+} channels in skeletal muscle fibers (Nánási et al., 1994; Benders et al., 1997) and isolated cardiac myocytes (Moreno-Sánches and Hansford, 1988). However, both in previous *in vivo* study (Cruz-Höfling et al., 2002), as in the current one, no electronmicrograph images suggestive of Ca^{2+} accumulation into the mitochondria was seen in veratrine-treated samples.

On the other hand, biochemical studies of isolated mitochondria showed that during changes in metabolic steady states of coupled mitochondria reversible ultrastructural transformations occurs in the organelles (Candipan and Sjöstrand, 1984a). Hackenbrock (1966) reported that depending on the respiration state mitochondria acquires a specific morphological configuration, such as reversibly expanded (orthodox) in state 4 respiration rate, reversibly condensed in state 3 respiration rate, and irreversibly swollen when respiration is uncoupled. A review of literature reveals that isolation media and methods of isolation such as digitonin treatment, sonic vibration and others are responsible for a great variability in morphology of the isolated mitochondria (Candipan and Sjöstrand, 1984b).

In this work, the electron microscopic analysis of the mitochondria pellets collected after realization of our biochemical assays demonstrated the presence of several mitochondria configurations that matched with those described elsewhere (Hackenbrock, 1966; Candipan and Sjöstrand, 1984a). Also, while morphological alterations were seen starting with 250 µg/ml veratrine concentration, a significant inhibition of oxygen consumption was only seen with 450 µg/ml. Veratrine and veratridine alkaloids also caused a dose-dependent decrease on respiratory parameters and dissipation membrane electrical potential supported by NADH and $FADH_2$-linked substrates in isolated rat skeletal muscle mitochondria (manuscript submitted).

Previous experiments showed that this alkaloid inhibits the respiration also on isolated mouse skeletal muscle mitochondria as seen in rats (data not shown).

These toxic effects of veratrine and veratridine could suggest a non-specific alteration on mitochondrial inner membrane permeability. The cytochemical reaction for dehydrogenases and COX did not exhibit positive activity for these enzymes mainly when mitochondria was swollen or presented ruptured membranes. We suggest that alteration on mitochondrial inner membrane permeability induced by veratrine could affect respiratory electron chain enzymes, thus changing activities of these enzymes.

Likewise veratrine, rhein (4,5-dihydroxyanthraquinome-2-carboxylic acid), a natural toxin extracted from traditional Chinese medicine herb (Lin et al., 2003), causes inhibition of the oxygen consumption (Kean, 1968), when respiration is stimulated by FCCP (carbonyl cyanide ρ-trifluoromethoxyphenylhydrazone) or ADP (Floridi et al., 1989). Scanning electron microscopy, TEM and electron paramagnetic resonance spectroscopy analyses indicated that cell surface and mitochondrial membranes would be direct targets in rhein-induced damage (Iosi et al., 1993).

Protamine is other example of inhibitor of the respiration that causes ultrastructural changes in isolated mitochondria (Popinigis et al., 1971; 1979). The changes in oxygen consumption by protamine were attributed to an increase on anion-conductivity of mitochondrial inner membrane, which would be a consequence of structural alterations caused by this protein on mitochondria (Popinigis et al., 1979).

The occurrence of swelling could be sign that there were configuration changes on mitochondrial inner membrane (Laiho and Trump, 1975) resulting in enhanced membrane

permeability. Some drugs and toxins are reported in literature as inducers of membrane permeability. For example, the polypeptide antibiotic duramycin interacts with specific lipids located on mitochondrial membranes inducing increase in membrane permeability of isolated rat heart mitochondria (Sokolove et al., 1989). Daphnetoxin, another example of isolated plant toxin (as for veratrine) altered oxygen consumption and induced swelling in isolated rat liver mitochondria (Peixoto et al., 2004). The authors concluded that mitochondrial changes resulted from non-specific membrane permeabilization induced by the toxin.

In the current study, the decrease of the oxygen consumption, changes in NADH-d, SDH and COX enzymes activities, most likely could be attributable to a direct interference of veratrine, a lipid-soluble alkaloid, in the phospholipid configuration of mitochondrial outer and inner membranes. Although osmotic unbalance caused by veratrine activation of Na^+ channels present in excitable sarcolemma could indirectly have affected mitochondrial morphology seen before (Freitas et al., 2001; Cruz-Höfling et al., 2002), the present study support the view that veratrine can act through a novel mechanism than sodium channels. Since mitochondria membranes do not possess voltage-dependent Na^+ channels (Bernardi, 1999), and since TTX pretreatment prevented both cell necrosis as well as mitochondrial alterations caused by i.m. injection of veratrine in mice (Freitas et al., 2001; Cruz-Höfling et al., 2002), a hypothesis that remains is that the alkaloid seems to be able to disturb the phospholipids configuration of mitochondria membrane, but not that of sarcolemma. The results do not permit to elucidate if the toxin enters or not into the organelle (or the muscle cell). It is necessary further investigation to elucidate the mechanism and the kind of phospholipids to which the alkaloid interacts, since is well known that there are differences in phospholipids of plasma membrane and mitochondrial membranes.

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LEGENDS TO FIGURES

Figure 1 – Electron micrographs of mitochondria from mice EDL (**A, C, E** and **G**) and SOL (**B, D, F** and **H**) muscle fibers. Controls (**A-B**): muscle mitochondria showing its typical structural organization. In EDL, mitochondria appeared arranged between myofibrils (**A**), while mitochondria from SOL were both intermyofibrillar and subsarcolemmal (**B**). Fifteen minutes after i.m. injection of 10 µg/ml veratrine (**C-D**), abnormal swollen (**s**) mitochondria (C), disrupted (**d**) or with areas devoid of cristae accompanied by electronlucent matrix (**e**), was only seen in SOL mitochondria (**D**). After 30 min, a huge increase of mitochondrial dimensions probably ought to fluid collection (**E-F**). Matrix electrolucency and disarray of cristae were observed on EDL muscle mitochondria (**e**). At 60 min, there was a visible recovery of mitochondria structure, but dimensions were not fully restored (**G-H**). SR= sarcoplasmic reticulum. Bar = 0.5 µm for all panels

Figure 2 - Electron micrographs of isolated mitochondria from hindlimbs muscles of rats exhibiting different patterns for NADH reaction. Controls (**A-B**)- Heterogeneously-sized spherical mitochondria (**A**): Note electrondense deposits of copper ferricyanide both in outer and inner membranes and in the intracristae spaces (**arrows**). Incubation with veratrine: 250 µg/ml (**C-D**) and 550 µg/ml (**E-F**)**:** There was mitochondria normally positive (**arrows**) for NADH reaction on membranes and cristae (**C**, **E** and **F**), and swollen mitochondria (*) with a thin deposit of NADH end reaction seen discontinuously labeled (**E**), or swollen mitochondria negative (**n**) for the enzyme reaction (**C-E**). Rare mitochondria, apparently with normal morphology, did not show any NADH reaction (**arrowheads**) with 550 µg/ml veratrine

concentration (**F**). Omission of NADH substrate on incubation medium gave negative reaction (control) (**G**). Bars = 1 µm (A, C-E, G); 0.5 µm (B, F).

Figure 3 – Electron micrographs of isolated mitochondria from rat hindlimbs muscle exhibiting different patterns of SDH reaction. Controls (**A-B**); Incubated with 250 µg/ml (**C-D**), or 550 µg/ml (**E-F**) veratrine. Negative control, with addition of 0.05 M sodium malonate (**G**). Note that the electrondense deposits of copper ferricyanide is seen both between the outer and inner mitochondrial membranes and within intracristae spaces (**arrows**). Swollen mitochondria devoid of cristae show negative reaction for SDH after incubation with veratrine (*). Bars = 0.5 µm (A-B, E, G); 1 µm (C-D, F).

Figure 4 – Electron micrographs of isolated rat muscle mitochondria stained for COX. Controls (**A-B**); Incubation with 250 µg/ml (**C-D**), and 550 µg/ml (**E-F**) veratrine. Negative control with addition of 0.01 M KCN (**G**). Electrondense deposits are observed on mitochondrial membranes and cristae, indicating COX activity (**arrows**). The majority of veratrine-treated mitochondria did not show positive COX reactivity, or the reaction was discontinuous (*). Bars = 0.5 µm (B-D, F); 1 µm (A, E, G).

Figure 5 – Percentage of diameters of isolated rat skeletal muscle mitochondria (mean values ± standard error) incubated and non-incubated with veratrine. There was no significant difference among treatments ($P < 0.05$).

Table 1 – Effects of veratrine on respiratory parameters of isolated rat skeletal muscle mitochondria. Each value represents the mean ± standard error of three different mitochondrial preparations. There were significant decrease on oxygen consumption (state 3), RCR and ADP/O in veratrine-treated isolated mitochondria in comparison to control.

*** P < 0.05,** P < 0.01 and *** P < 0.001**

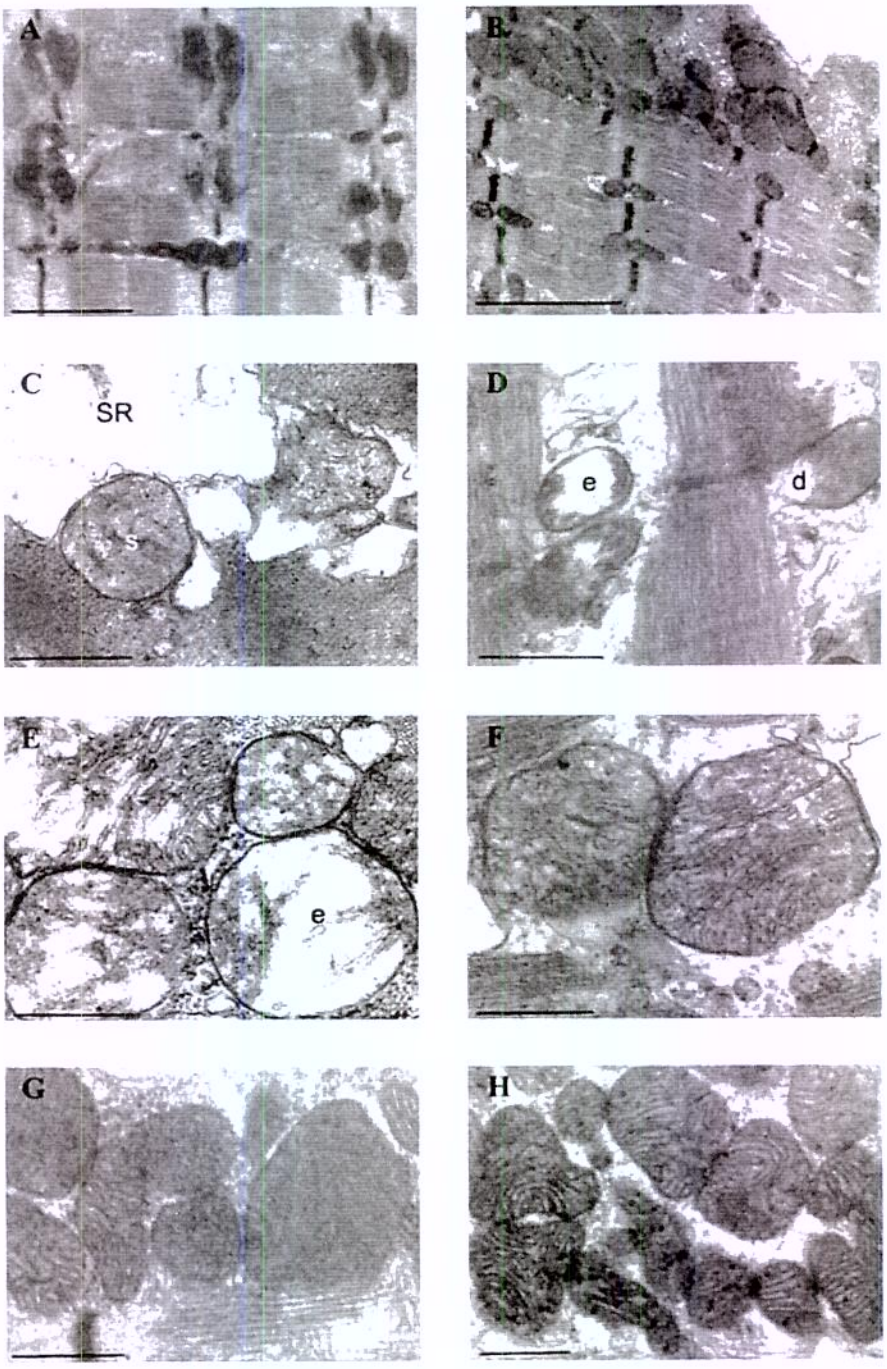


FIGURE 1
Freitas et al., 2006

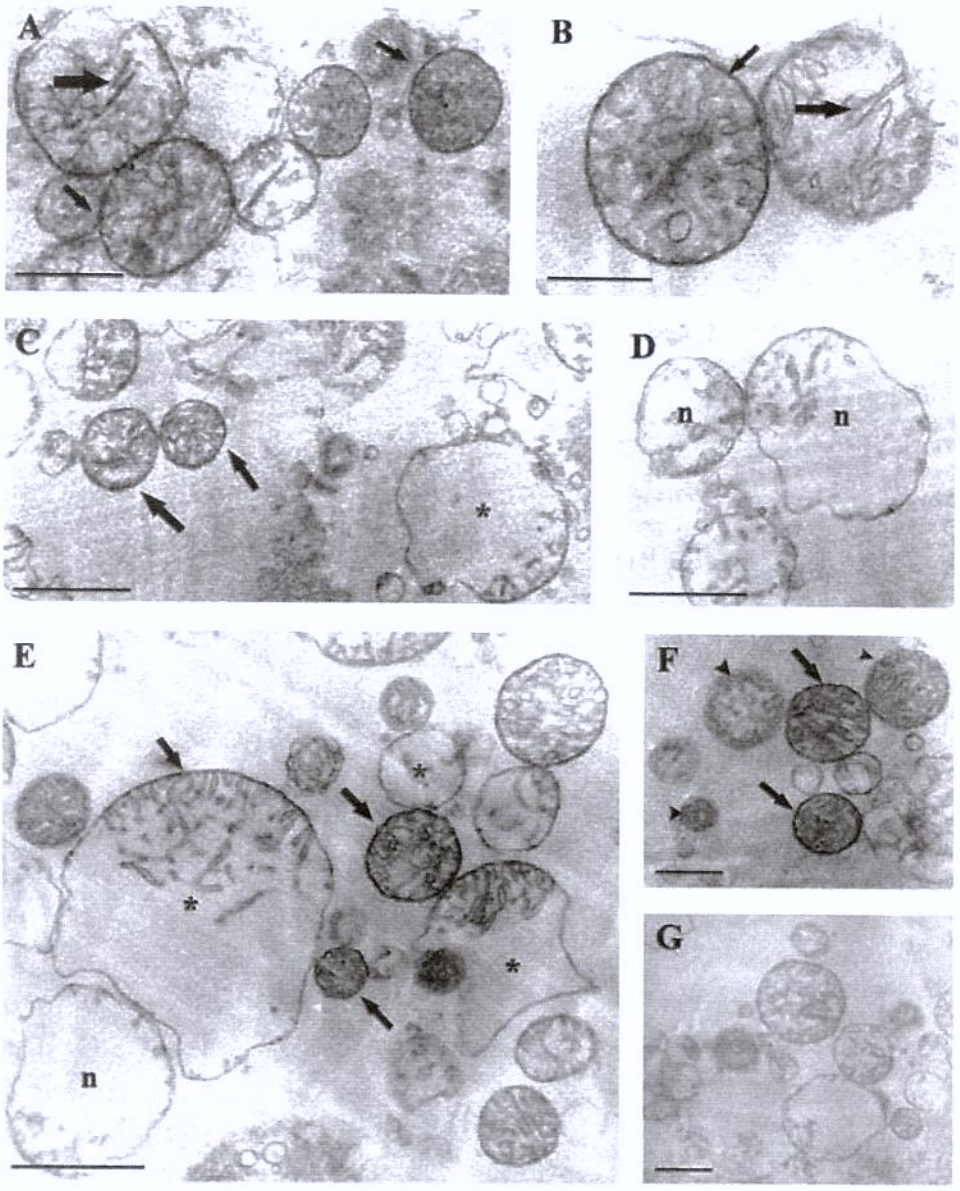
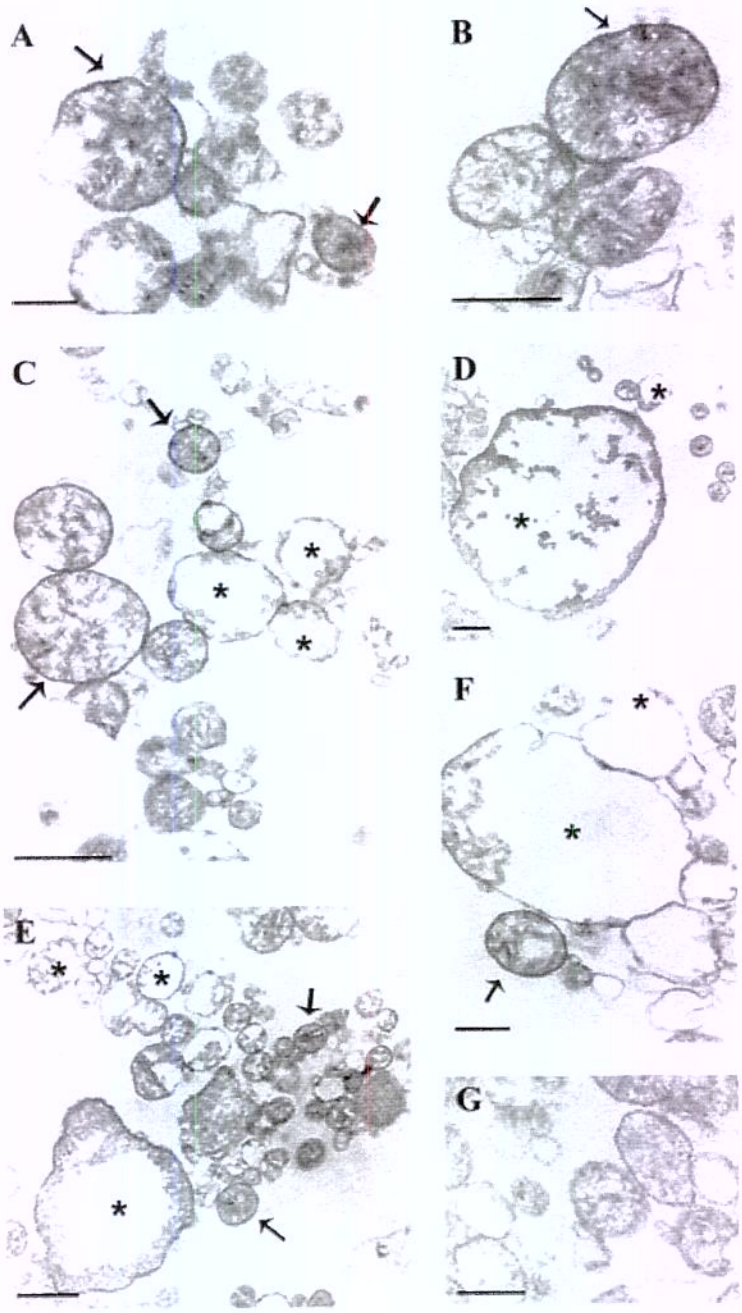


FIGURE 2
Freitas et al., 2006



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Freitas et al., 2006

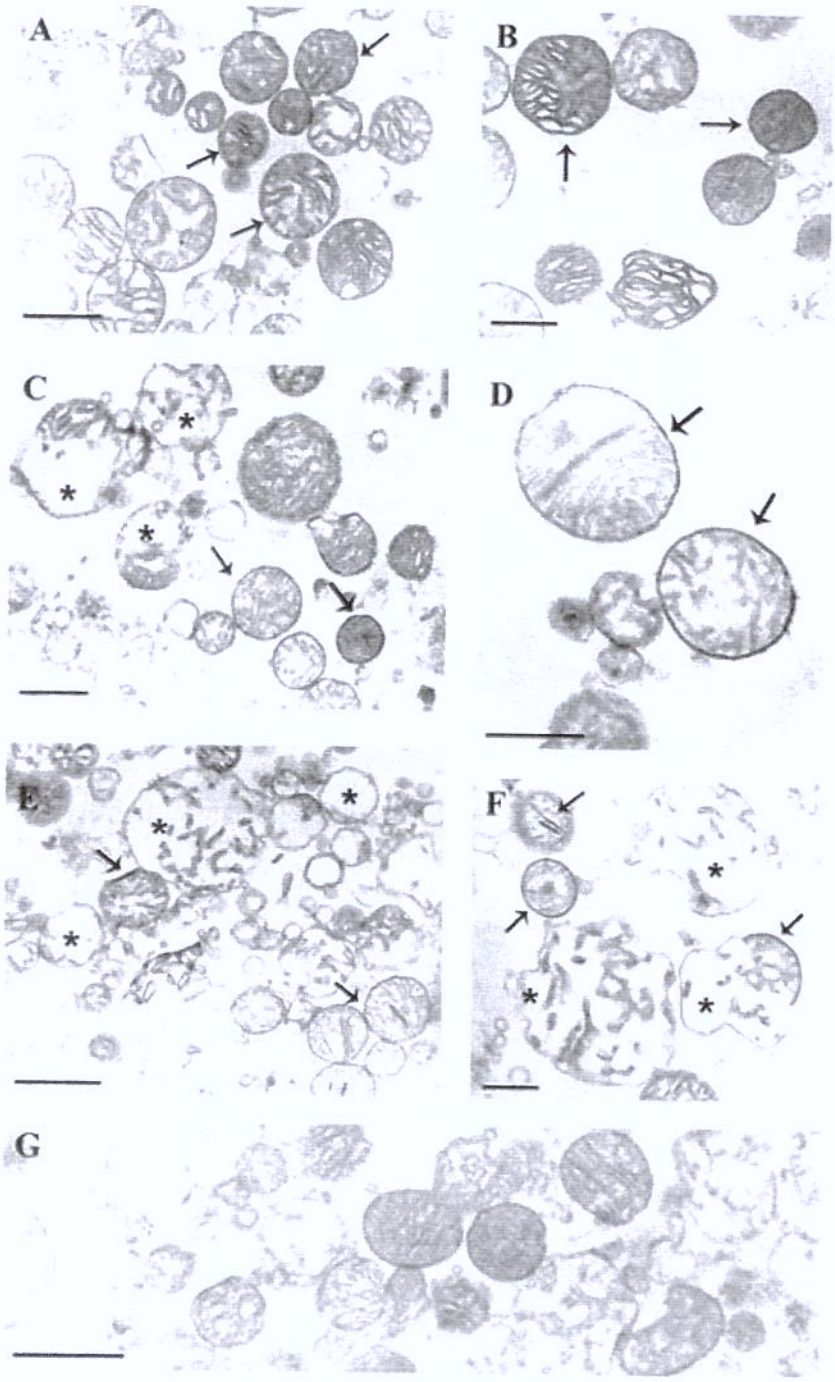

FIGURE 4
Freitas et al., 2006

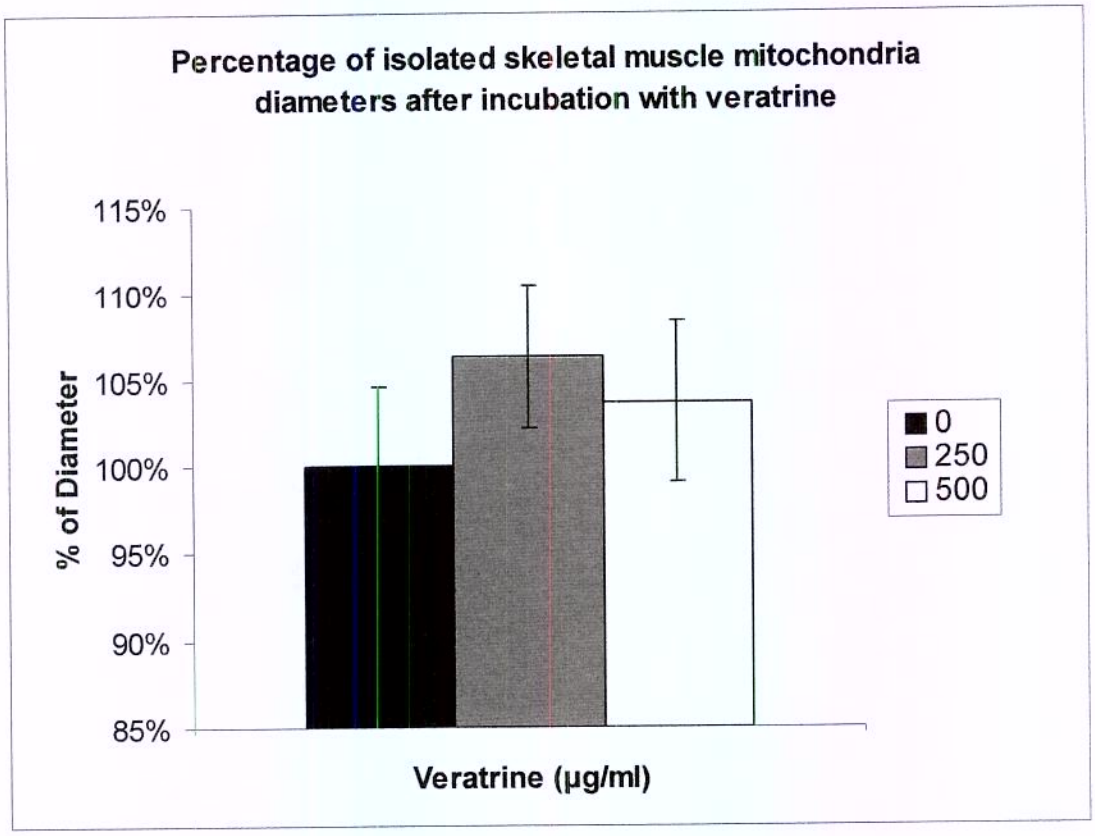

Figure 5

Freitas et al., 2005

VERATRINE (µg/ml)	STATE 3 (natoms O/min/mg)	STATE 4 (natoms O/min/mg)	RCR	ADP/O
0	160.9 ± 7.5	34.8 ± 1.8	4.7 ± 0.2	2.7 ± 0.1
250	143.8 ± 0.9	34.2 ± 0.7	4.2 ± 0.1	2.3 ± 0.1 **
350	136.8 ± 1.6	33.6 ± 1.4	4.1 ± 0.2	2.3 ± 0.1 **
450	124 ± 5.7 *	34 ± 1.8	3.7 ± 0.3	2.3 ± 0.1 **
550	111.1 ± 5.4 ***	32.1 ± 2.1	3.5 ± 0.3 *	2.3 ± 0.2 *

Table 1

Freitas et al., 2005

ARTIGO 2: EFFECTS OF VERATRINE AND VERATRIDINE ON OXYGEN CONSUMPTION AND ELECTRICAL MEMBRANE POTENTIAL OF ISOLATED RAT SKELETAL MUSCLE AND LIVER MITOCHONDRIA

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EFFECTS OF VERATRINE AND VERATRIDINE ON OXYGEN CONSUMPTION AND ELECTRICAL MEMBRANE POTENTIAL OF ISOLATED RAT SKELETAL MUSCLE AND LIVER MITOCHONDRIA

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Abstract

We have previously shown that veratrine, a mixture of alkaloids known as *Veratrum* alkaloids, produces skeletal muscle toxicity, and there is evidence that veratrine interferes with the energetics of various systems, including cardiomyocytes and synaptosomes. In this work, we explored the effects of veratrine and veratridine, a component of this mixture, in rat skeletal muscle mitochondria and compared the results with those seen in liver mitochondria. Veratrine and veratridine alkaloids caused a significant concentration-dependent decrease in the rate of state 3 respiration, respiratory control (RCR) and ADP/O ratios in isolated rat skeletal muscle mitochondria (RMM), but not in rat liver mitochondria (RLM) supported by either NADH-linked substrates or succinate. The oxygen consumption experiments showed that RMM were more susceptible to the toxic action of *Veratrum* alkaloids than RLM. The addition of veratrine (250 μg/ml) to RMM caused dissipation of the mitochondrial electrical membrane potential during succinate oxidation, but this effect was totally reversed by adding ATP. These results indicate that there are chemical- and tissue-specific toxic effects of veratrine and veratridine on mitochondrial respiratory chain complexes. Identification of the specific respiratory chain targets involved should provide a better understanding of the molecular mechanisms of the toxicity of these agents.

Key words: Isolated mitochondria, mitochondrial membrane potential, oxygen consumption, respiratory chain complex, veratrine, veratridine

1. Introduction

Veratrine is a commercial mixture of alkaloids extracted from the plant *Schoenocaulon officinale* A. Gray, ex Benth, 1840 (or *Veratrum officinale* Schl. & Cham, 1831) and other species of the family Liliaceae, and contains principally two esters, cevadine and veratridine (Benforado, 1967; Catterall, 1980; Ulbricht, 1998). Chemically, these alkaloids are lipid-soluble compounds with a steroid structure containing a secondary or tertiary nitrogen atom within a ring (Benforado, 1967). Pharmacological and electrophysiological studies have shown that veratrine causes persistent depolarization of excitable membranes by activating voltage-dependent Na^+ channels which increases the Na^+ permeability (Ulbricht, 1969, 1998; Vital Brazil and Fontana, 1985; Sutro, 1986) and influx of Ca^{2+} via the Na^+-Ca^{2+} exchanger (Wermelskirchen et al., 1992).

Our previous ultrastructural and histochemical studies demonstrated that the intramuscular injection of veratrine induced degenerative changes in mitochondria that included swelling, cristae deformation and alteration of the contractility and metabolic activity of muscle fibers in fish and mammals (Freitas et al., 2001, 2002; Cruz-Höfling et al., 2002). In the 19th century, veratrine was used therapeutically to hypertension because of its ability to decrease blood pressure and heart rate, a phenomenon known as the "Bezold-Jarisch effect" (Krayer, 1961). However, the side effects provoked by veratrine limited its clinical use (Benforado, 1967). Reports of human and cattle poisoning following the ingestion of leaves from *Veratrum* plants (Benforado, 1967; Jaffe et al., 1990) have described symptoms similar to those caused by bioenergetic poisons (Wallace and Starkov, 2000). In addition, veratrine is teratogenic in rats, mice and hamsters (Keeler, 1975; Omnell et al., 1990).

Experiments *in vitro* have shown that veratridine stimulates oxygen consumption in isolated cardiac myocytes and synaptosomes (Moreno-Sánchez and Hansford, 1988; Wermelskirchen et al., 1992) and induces apoptotic cell death in cultured neuronal and chromaffin cells (Jórdan et al., 2000; Callaway et al., 2001). Furthermore, veratrine increases the synthesis of nitric oxide and, consequently, the levels of cyclic GMP via the activation of L-arginine-NO pathway (Lizasoain et al., 1995; Tsai et al., 1999).

In view of the variety of effects discussed above, identification of the intracellular targets responsible for the toxicity of *Veratrum* alkaloids is of particular interest. In this study, we examined the effects of *Veratrum* alkaloids on the bioenergetics of isolated rat skeletal muscle and liver mitochondria.

2. Material and methods

2.1. Animal care

Age-matched Wistar rats (*Rattus novergicus albinos*) of both sexes (two months old: 200-250 g) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP and were housed at 20-22°C on a 12 h light/dark cycle, with free access to a standard diet (Labina/Purina, Campinas, SP, Brazil) and tap water. The experiments described here were approved by the institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, protocol no. 487-1) and were done in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised in 1996).

2.2. Chemicals

Veratrine hydrochloride was dissolved in distilled water adjusted to pH 7.4 with KOH and veratridine was dissolved in ethanol. ADP, antimycin A, ATP, bovine serum albumin (BSA), ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid) (EGTA), ethylenediaminetetraacetic acid (EDTA), FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), glutamate, α-ketoglutarate, malate, oligomycin, pyruvate, rotenone, safranin O, succinate, Tris [Tris(hydroxymethyl)aminomethane], veratrine and veratridine were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Isolation of rat skeletal muscle mitochondria (RMM)

Wistar rats (female and male) were killed by cervical dislocation before the hind limb muscles were removed for the isolation of mitochondria by the method of Tonkonogi and

Sahlin (1997), with modifications. Briefly, the muscles were dissected, perforated and immersed in ice-cold isolation buffer of the following composition: 100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, pH 7.4, 1 mM K_2HPO_4, 0.1 mM EGTA and 0.2% BSA, at 4°C. Subsequently, the muscles were washed in ice-cold medium, minced finely with scissors and homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland), at a rheostat setting of 4, and then with a Potter-Elvehjem-type homogenizer. The homogenate was centrifuged at 700 *g* for 10 min at 4°C and the supernatant was retrieved and centrifuged at 10,000 *g* for 10 min. The resulting pellet was then resuspended in the same buffer and recentrifuged at 6,000 *g* for 3 min.

The final mitochondrial pellet was resuspended in medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA to a protein concentration of 30-40 mg/ml and kept on ice during all of the biochemical assays. Protein concentrations were determined by the biuret method using BSA as standard (Gornall et al., 1949).

2.4. Isolation of rat liver mitochondria (RLM)

RLM were isolated by conventional differential centrifugation from adult female Wistar rats fasted overnight. The buffer used consisted of 10 mM Hepes-K^+, pH 7.2, containing 250 mM sucrose and 0.5 mM EGTA. The mitochondria were washed and resuspended in the same buffer without EGTA. Protein concentrations (80-100 mg/ml) were determined by the biuret method.

2.5. Oxygen uptake measurements

Oxygen consumption was measured with a Clark-type electrode (Yellow Springs Instruments Co., OH, USA) in 1.3 ml of reaction medium (28°C), in a sealed glass cuvette equipped with a magnetic stirrer. Two reaction media were used to measure oxygen consumption in the RLM and RMM mitochondrial preparations. Medium I consisted of 10 mM Tris-HCl buffer, pH 7.4, 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM K_2HPO_4, 0.1 mM EDTA, whereas medium II consisted of 10 mM Hepes-K^+ buffer, pH 7.2, 125 mM sucrose, 65 mM KCl, 0.5 mM EGTA, 2 mM K_2HPO_4 and 1 mM $MgCl_2$. RMM respiration gave better results with reaction medium I in the biochemical assays, whereas RLM respiration was equally good in both media. In some experiments, different concentrations of veratrine were added to the reaction medium before the addition of isolated mitochondria and substrate. Other additions were made as indicated in the figure legends.

The respiratory control ratio (RCR = state 3 respiration/state 4 respiration), the ADP/O ratio (number of ADP molecules added to the medium per oxygen atom consumed during phosphorylation), and the respiratory rates were calculated according to Chance and Williams (1956).

2.6. Mitochondrial membrane electrical potential measurements ($\Delta\Psi m$)

The mitochondrial electrical membrane potential was estimated based on the fluorescence changes of 5 μM safranin O (excitation-emission, 495-586 nm) recorded on a Hitachi F-4010 spectrofluorometer (Hitachi Ltd., Tokyo, Japan), under the same conditions as used to measure oxygen consumption.

2.7. Statistical analysis

The results were expressed as the mean + S.E.M. of the number of experiments indicated. Statistical comparisons of the results for the biochemical assays were done using one-way analysis of variance followed by the Bonferroni test for multiple comparisons. A p value of 0.05 indicated significance.

3. Results

3.1. Inhibition of oxygen consumption by veratrine and veratridine

Veratrine exerted a concentration-dependent adverse effect on mitochondrial respiration supported by NADH-linked substrates (Figs. 1 and 2). The respiratory parameters confirmed the good coupling of RMM (RCR = 4.5 ± 0.2, n=3) (Fig. 1). At concentrations ≥ 450 μg/ml, veratrine caused a progressive decrease in the RCR, with a maximum inhibition of 64% at 1000 μg/ml compared to the control ($P < 0.05$) (Fig. 1). A veratrine concentration of 250 μg/ml significantly reduced the ADP/O ratio by 15% (Fig. 1). Figure 2 shows a significant decrease in the state 3 respiration rate ($P < 0.05$) at 450 μg of veratrine/ml; overall, there was 23% to 63% inhibition with the different concentrations of veratrine studied. The state 4 respiration rate was not significantly affected by veratrine compared to the control (Fig. 2). Representative traces of oxygen consumption are shown in Figure 3.

When compared with the results obtained for RMM, the respiratory parameters of RLM were unaffected by veratrine, regardless of the reaction medium (I or II) used (Figs. 4,5 and 6). Inhibition of the RCR varied from 7% to 14.5%, depending on the veratrine concentration, whereas the ADP/O ratios was unaltered by the addition of veratrine to the reaction medium (Fig. 4). As with the RCR, the state 3 respiration rate tended to decrease after addition of the alkaloid, although this change was not significant (Fig. 5). In RLM, veratrine did not significantly inhibit the state 4 respiration rate (Fig. 5). Representative traces of these experiments are shown in Figure 6.

Figure 7 shows the inhibitory effects of veratrine on FCCP-uncoupled respiration supported either by NADH-linked substrates or succinate. The inhibition of oxygen

consumption was concentration-dependent and was 14%, 11% and 23.7% at 250, 500 and 1000 µg of veratrine/ml for Complex I substrate cocktails, respectively ($P < 0.05$). The respiration supported by succinate was significantly inhibited only by 500 µg of veratrine/ml (30% inhibition compared to control) (Fig. 7).

Veratridine also caused a concentration-dependent decrease in oxygen consumption at lower concentrations than obtained with the veratrine mixture (Figs. 8 and 9). Figure 8 shows that the RCR was inhibited 27%, 38% and 51% by 50, 150 and 250 µg of veratrine/ml, respectively. The ADP/O ratio was inhibited from 14% to 39% by these same veratrine concentrations (Fig. 8). Veratrine reduced the state 3 respiration in a manner similar to that for the RCR (Fig. 9). In contrast, the state 4 respiration rate tended to increase in the presence of veratrine, particularly at a veratrine concentration of 250 µg/ml (22% increase compared to the control) (Fig. 9).

3.2. Effects of veratrine on mitochondrial electrical membrane potential ($\Delta\Psi m$)

Figure 10 shows that 250 µg of veratrine/ml significantly reduced the $\Delta\Psi m$ in RMM and that this reduction was totally reversed by ATP. This finding indicated that i) ATP hydrolysis by the ATP synthase was unaffected by veratrine and that ii) at this concentration, veratrine did not increase the permeability of the inner membrane of muscle mitchondria. The subsequent addition of oligomycin and FCCP sequentially and cumulatively eliminated the $\Delta\Psi m$, thus supporting the foregoing conclusions. In contrast, 250 µg of veratrine/ml did not significantly alter the $\Delta\Psi m$ of RLM (data not shown).

4. Discussion

As shown here, veratrine caused concentration-dependent inhibition of state 3 respiration (phosphorylating respiration) and decreased the RCR and ADP/O ratios of respiration supported by NADH- or $FADH_2$-linked substrates in isolated rat skeletal muscle mitochondria. Veratridine, one of the principal components of the veratrine mixture (Benforado, 1967), exerted a similar action on mitochondrial respiration, thus supporting the notion that this compound is responsible for the effects seen with the veratrine mixture. In contrast to these findings, veratridine increases oxygen consumption in isolated cardiac myocytes and synaptosomes, probably through the action of this alkaloid on the Na^+-Ca^{2+} exchanger, with a resulting increase in the cytosolic Na^+ (Moreno-Sánchez and Hansford, 1988; Wermelskirchen et al., 1992). Our results also show that veratrine selectively affects the oxygen consumption of muscle mitochondria, without affecting that of isolated liver mitochondria. Liver mitochondria have long been a paradigm in studies of bioenergetics and were used here to allow comparison with muscle mitochondria. The reasons for this difference in selectivity in these two mitochondrial systems remain to be determined.

Various studies have shown tissue-specific differences in the properties of isolated mitochondria. For example, mitochondria isolated from heart, liver, skeletal muscle and brain vary in their sensitivity to agents capable of inducing the inner membrane permeability transition (MPT). Higher concentrations of calcium are required to induce the MPT in heart mitochondria than in liver mitochondria (Palmer et al., 1981; Novgorodov et al., 1992). In addition, isolated brain mitochondria are more resistant to the MPT in conditions that rapidly induce the opening of the inner membrane permeability pore in liver mitochondria (Berman et al., 2000). This resistance has been ascribed to differences in the expression of certain proteins

in liver and brain mitochondria, such as creatine kinase (O'Gorman et al., 1997; Beutner et al., 1998) and members of the bcl-2 family of proteins (Reed et al., 1998), which can activate or inhibit the permeability transition. A further difference involves the pathways for Ca^{2+} release from mitochondria. The efflux of calcium from mitochondria in excitable tissues (brain and skeletal muscle) is primarily through sodium-dependent transport, whereas in liver this release is essentially independent of sodium (Deryabina et al., 2004). The biochemical and physiological differences among mitochondria from diverse sources could explain the different sensitivities of liver and skeletal muscle mitochondria seen here after incubation with veratrine.

Selectivity in the effects of veratrine was also seen in our earlier experiments in vivo which showed that veratrine significantly increased the percentage of type I (oxidative) and type IIA (oxidative-glycolytic) fibers in *extensor digitorum longus* (EDL), but decreased the proportion of type I fibers, without changing that of type IIA fibers, in *soleus* muscle (Freitas et al., 2002). Hence, the intrinsic metabolic, contractile and biochemical characteristics of the muscle (predominantly fast-twitch glycolytic and predominantly slow-twitch oxidative, respectively) may influence the response of the same fiber type to veratrine, as shown by the m-ATPase reaction. These findings offer an interesting line of investigation for exploring the toxic effects of veratrine and veratridine on isolated mitochondria of predominantly oxidative and predominantly glycolytic muscles.

Mitochondrial respiration in rabbits is regulated by Ca^{2+}-activated m-ATPase and creatine and adenylate kinases, depending on the fiber type (Gueguen et al., 2005). These authors showed that type I and IIA fibers displayed a specific regulation of mitochondrial respiration that was characterized by a reduced affinity for ADP and functional coupling

between the two kinases indicated above and oxidative phosphorylation. We have recently shown that the NADH dehydrogenase, succinic dehydrogenase and cytochrome oxidase activities of muscle mitochondria isolated from rat hind limbs are decreased after incubation with veratrine (Freitas et al., 2006). The decrease in the metabolic activity of rat skeletal muscle fibers seen in vitro may help to explain the inhibitory action of veratrine on oxidative phosphorylation. In agreement with this, veratrine depresses the mitochondrial electrical membrane potential during succinate oxidation via a mechanism independent of an uncoupling action since ATP hydrolysis totally reversed this effect (Castelli et al., 2005).

One possible explanation for the effects of veratrine on mitochondrial bioenergetics may be related to the solubility of this alkaloid in the inner mitochondrial membrane. The inner mitochondrial membrane is anisotropic with the matrix side, is negatively charged and can accumulate large amounts of positively charged lipophilic substances and some acids (Wallace and Starkov, 2000). Veratridine is a lipophilic alkaloid and a weak base (pK 9.5-9.7). After incubation with veratridine solution at pH 7.3, there is an increase in the protonated form of this compound inside mitochondrial matrix (Honerjäger et al., 1992), and it could induce depolarization of the inner mitochondrial membrane. Based on membrane electrical potential measurements, it is suggested that veratrine might act as protonated form on mitochondrial matrix like the veratridine, justifying the decrease of the membrane electrical potential upon addition of the alkaloid. After veratrine incubation, the percentage of inhibition on oxygen consumption was higher when the respiration was coupled, than it was uncoupled by FCCP, suggesting the need for an electrical potential in order for veratrine to interact with the mitochondrial membrane. A similar protonophoric mechanism of action was proposed for the antitumoral alkaloid ellipticine that, at low concentrations, uncoupled oxidative

phosphorylation, but at high concentrations, inhibited the electron transport chain at the level of cytochrome *c* oxidase (Schwaller et al., 1995).

In conclusion, our results suggest that the toxicity of veratrine and veratridine (a naturally occurring sodium channel-activating alkaloid) in isolated rat skeletal muscle mitochondria may be related to an inhibitory action on the respiratory chain through interaction of the alkaloids with the mitochondrial inner membrane. Since this inhibition was not seen in rat liver mitochondria, we conclude that these alkaloids have a tissue-selective capacity to disturb mitochondrial bioenergetics.

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FIGURE LEGENDS

Fig. 1. Effects of veratrine on the RCR and ADP/O ratios of isolated rat skeletal muscle mitochondria. Different concentrations of veratrine were added to the reaction medium I (1.3 ml, 28°C), as described in Section 2.5, followed by the addition of mitochondria (0.3 mg/ml) and incubation for 1 min. After this incubation, respiration was initiated by adding a Complex I substrate cocktail (5 mM α-ketoglutarate, 5 mM malate, 5 mM glutamate and 5 mM pyruvate). The state 3 respiration rate was measured in the presence of 200 nmol of ADP and state 4 respiration was measured after exhaustion of the ADP. The control represents an experiment without the addition of veratrine to the reaction medium. Each value represents the mean ± S.E.M. of three different mitochondrial preparations. *$P<0.05$, compared to the control.

Fig. 2. Effects of veratrine on state 3 and state 4 respiration of isolated rat skeletal muscle mitochondria. Mitochondria (0.3 mg/ml) were incubated for 1 min with different concentrations of veratrine in the reaction medium (1.3 ml, 28°C). Other additions: 5 mM of Complex I substrate cocktail and 200 nmol of ADP. Each point represents the mean ± S.E.M. of three different mitochondrial preparations. *$P<0.05$, compared to the control.

Fig. 3. Representative traces showing the oxygen consumption from isolated rat skeletal muscle mitochondria. The experiment was performed in the same conditions as for Figures 1 and 2. Additions of RMM (0.3mg/ml) and ADP (200 nmol) are indicated by arrows. Each rate

of oxygen consumption represents the mean ± S.E.M. (nanoatoms oxygen/min/mg) of three different mitochondrial preparations. (A) Control without veratrine incubation; RMM incubated with 550 μg/ml (B) and 1000 μg/ml (C) veratrine.

Fig. 4. Effects of veratrine on the RCR and ADP/O ratios of isolated rat liver mitochondria. Mitochondria (0.5 mg/ml) were incubated for 1 min in the absence or presence of veratrine in the reaction medium II (1.3 ml, 28°C). Other additions: 5 mM succinate, 2 μM rotenone and 200 nmol of ADP. Each point represents the mean of 2-3 different mitochondrial preparations.

Fig. 5. Effects of veratrine on state 3 and state 4 respiration from isolated rat liver mitochondria. Mitochondria (0.5 mg/ml) were incubated for 1 min with different concentrations of veratrine in the reaction medium II. Other additions: 5 mM succinate, 2 μM rotenone and 200 nmol of ADP. Each point represents the mean of 2-3 different mitochondrial preparations.

Fig. 6. Representative traces showing the oxygen consumption from isolated rat liver mitochondria. The reaction medium I (1.3 ml, 28°C) used in this experiment contained Complex I substrate cocktail. Additions of RLM (0.3mg/ml) and ADP (200 nmol) are indicated by arrows. Each rate of oxygen consumption represents the mean ± S.E.M. (nanoatoms oxygen/min/mg) of three different mitochondrial preparations. (A) Control without veratrine incubation; (B) RLM incubated with 1000 μg/ml veratrine.

Fig. 7. Inhibition of FCCP-stimulated respiration by veratrine. The rate of oxygen consumption by uncoupled mitochondria was measured as a function of the alkaloid concentration. Isolated rat skeletal muscle mitochondria (0.3 mg/ml) were added to a reaction medium I (1.3 ml, 28°C) containing 0.5 μM FCCP plus a Complex I substrate cocktail or 4 mM succinate and 2 μM rotenone ($n \geq 5$). Each point (mean ± S.E.M.) is significantly different from the control (*$P < 0.05$).

Fig. 8. Effects of veratridine on the RCR and ADP/O ratios of isolated rat skeletal muscle mitochondria. Mitochondria (0.3 mg/ml) were incubated for 1 min with different concentrations of veratridine in the reaction medium I (1.3 ml, 28°C). After this incubation, oxygen consumption was initiated by adding 5 mM of a Complex I substrate cocktail and phosphorylating respiration was measured in the presence of 200 nmol of ADP. Each point represents the mean of 2-3 different mitochondrial preparations.

Fig. 9. Effects of veratridine on state 3 and state 4 respiration of isolated rat skeletal muscle mitochondria. Mitochondria (0.3 mg/ml) were incubated for 1 min with different concentrations of veratridine in the reaction medium I. Other additions: 5 mM Complex I substrate cocktail and 200 nmol of ADP. Each point represents the mean of 2-3 different mitochondrial preparations.

Fig. 10. Effect of veratrine on mitochondrial electrical membrane potential ($\Delta\Psi m$). Isolated rat skeletal muscle mitochondria (0.3 mg/ml) were added to a reaction medium I (2 ml, 28 °C)

containing 4 mM succinate, 2 μM rotenone and 5 μM safranin O. Veratrine (250 μg/ml), ATP (0.5 mM), oligomycin (1 μg/ml) and FCCP (1 μM) were added where indicated. The trace is representative of at least three experiments done using different mitochondrial preparations.

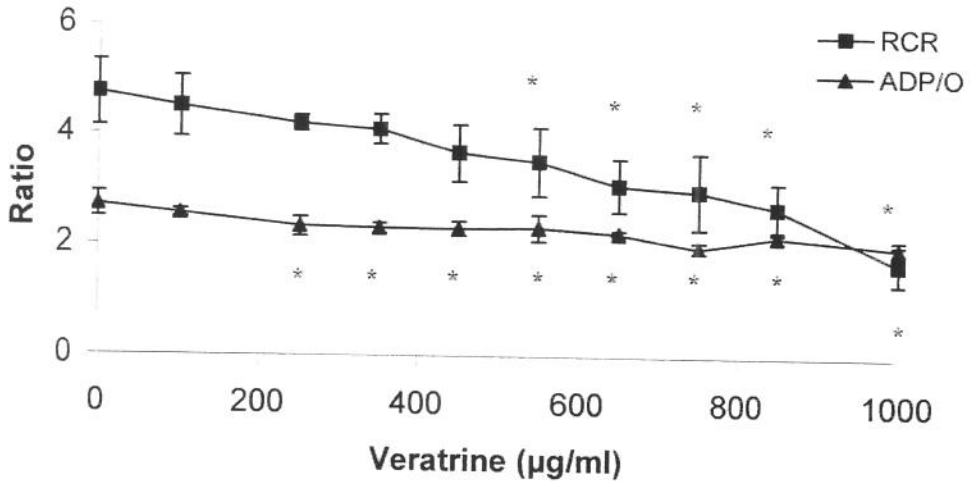


FIGURE 1: Freitas et al., 2006

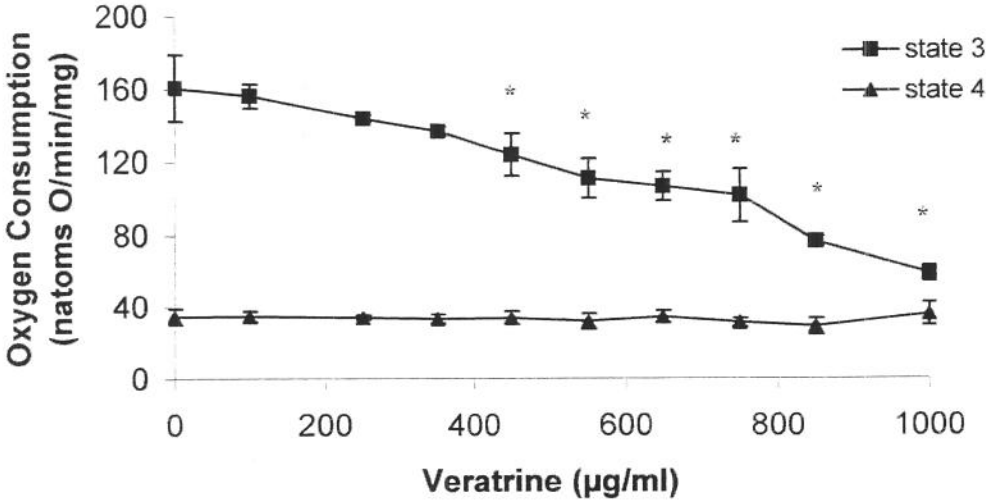


FIGURE 2: Freitas et al., 2006

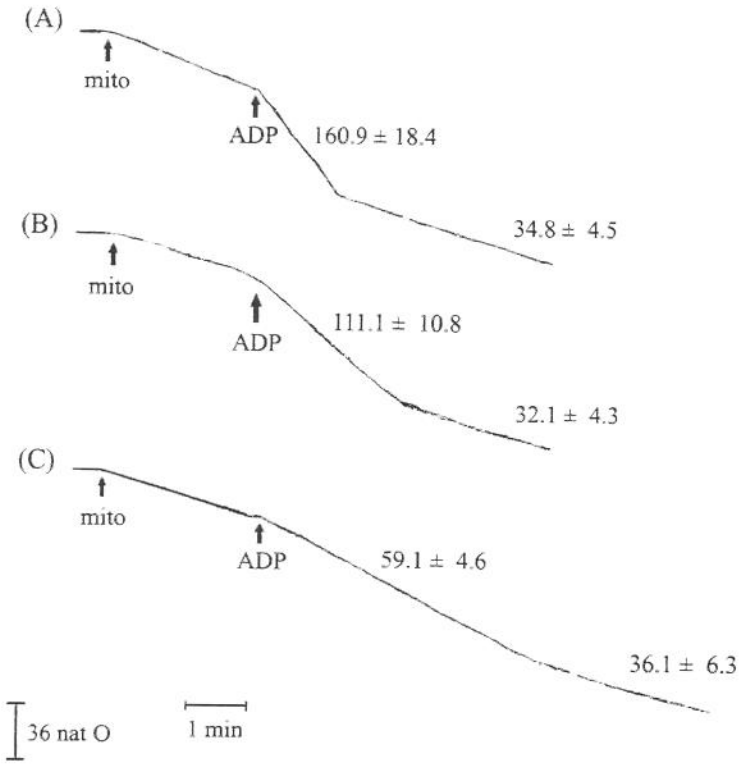
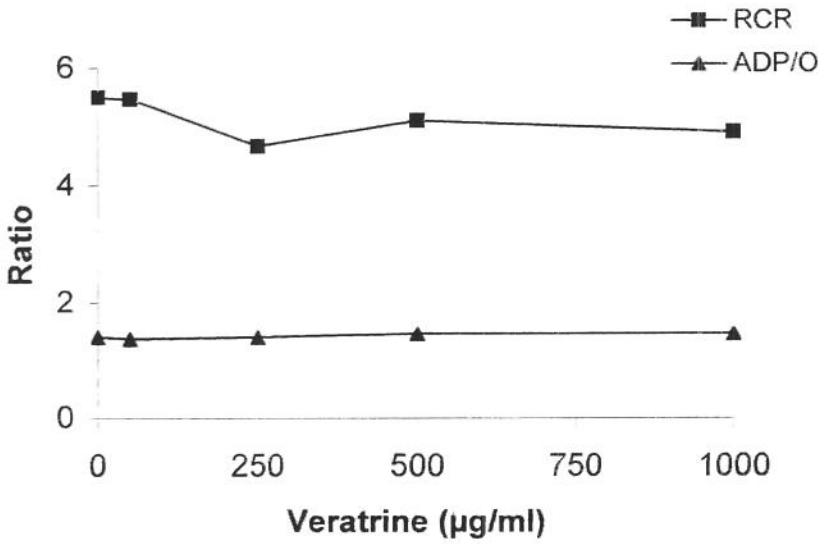


FIGURE 3: Freitas et al., 2006



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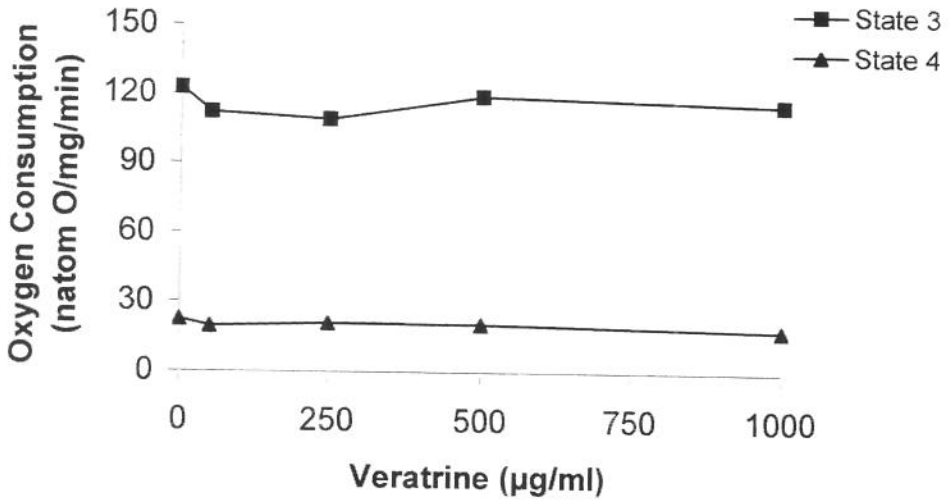

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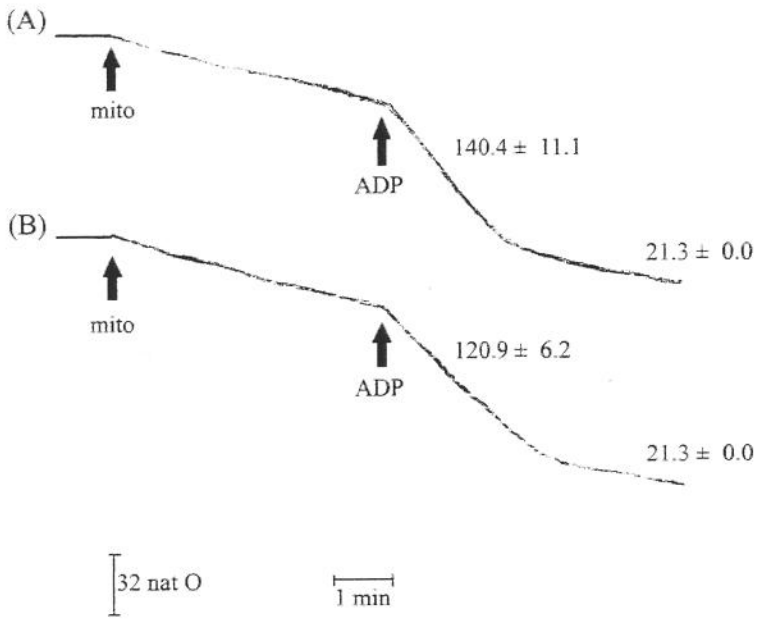

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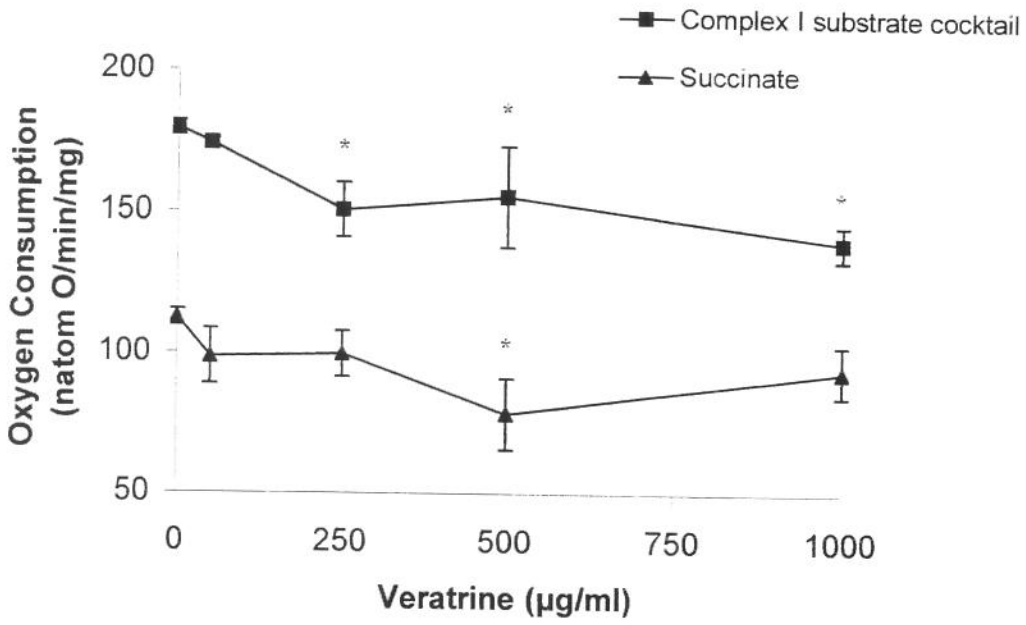

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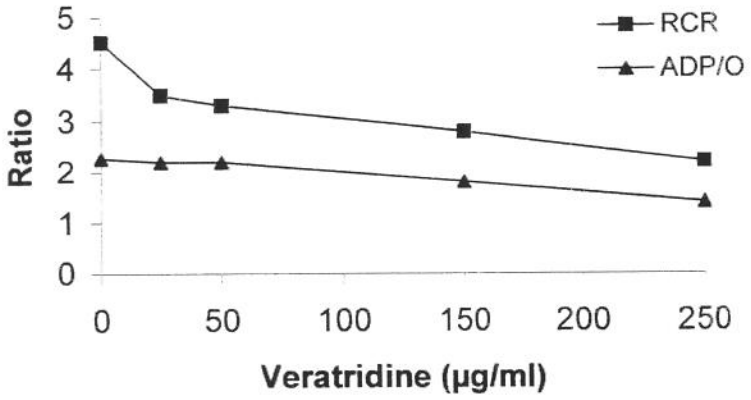

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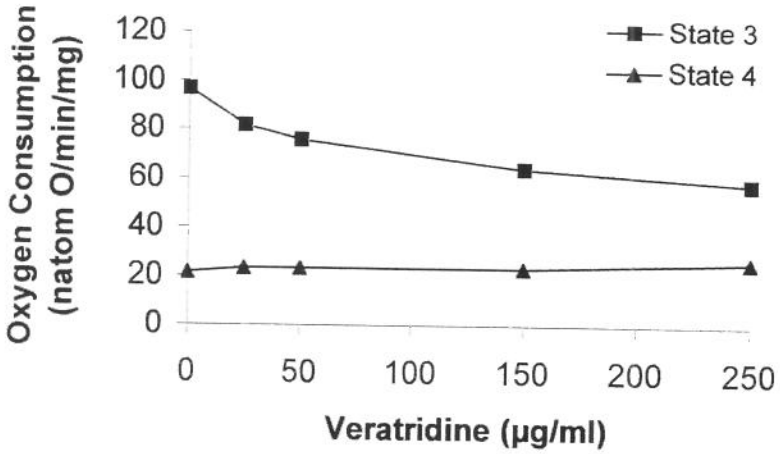

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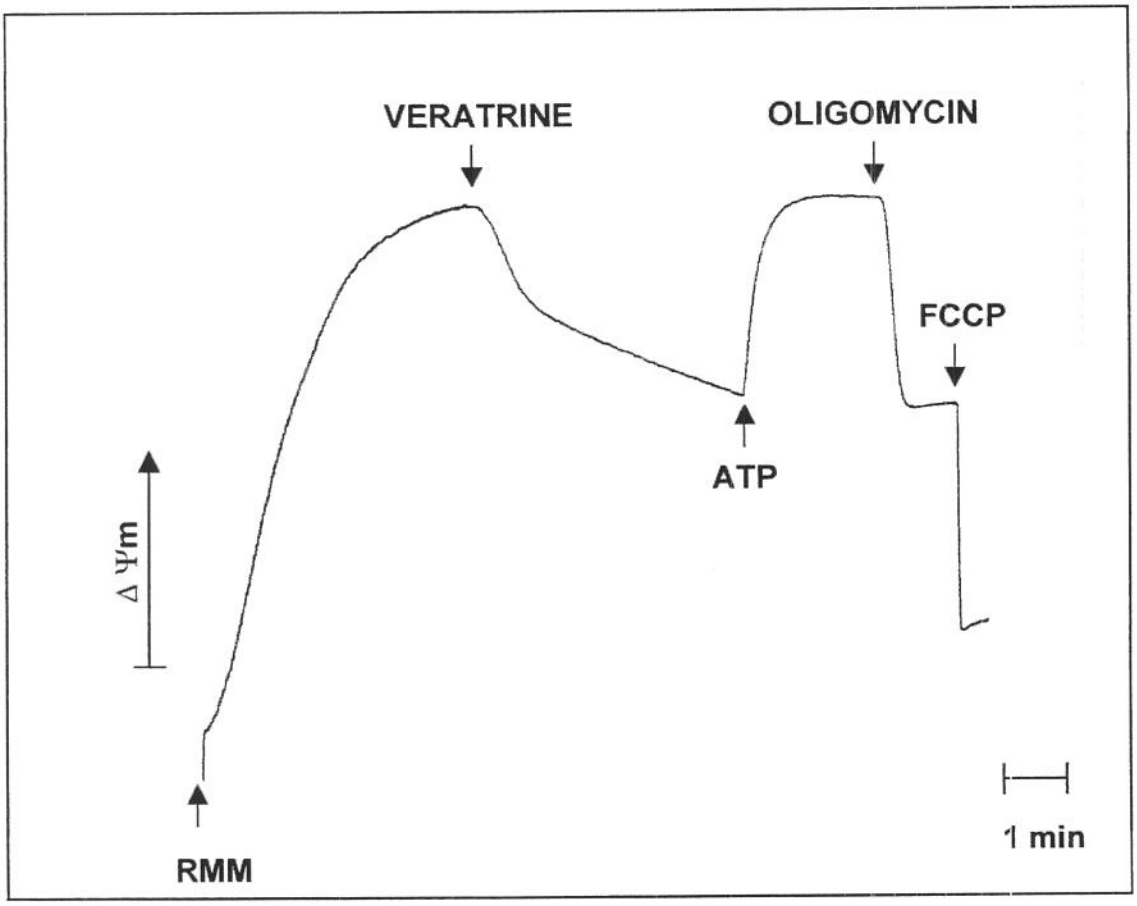

FIGURE 10: Freitas et al. 2006

ARTIGO 3: MITOCHONDRIA AND SARCOPLASMIC RETICULUM AS CELLULAR TARGETS FOR VERATRINE

MITOCHONDRIA AND SARCOPLASMIC RETICULUM AS CELLULAR TARGETS FOR VERATRINE

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Running Title: Mitochondria and sarcoplasmic reticulum are targets veratrine

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Abstract

Ultrastructural changes in skeletal muscle fibers, myelinated axons, and neuromuscular junctions caused by toxins that induced persistent activation of sodium channels have been reported by several authors. In the present study, isolated mouse phrenic nerve-diaphragm preparations were incubated for 60 or 120 min in Tyrode solution containing 5 or 10 µg/mL of veratrine and the effects of such treatment on the morphology of the muscle and on the twitch tension were examined. In addition, the action of veratrine (5 µL - 10 µg/mL) was studied in mouse *extensor digitorum longus* (EDL) and *soleus* (SOL) muscles and in the lateral muscle from freshwater teleost *Oreochromis niloticus* after 15 to 60 min of intramuscular injection. Pretreatment with tetrodotoxin (TTX) (1 µg/mL) was employed in order to determine if the veratrine-induced alterations were due to sodium channel activation. Veratrine greatly affected the morphology of a number of muscles cells, which presented rows of enlarged cisterns resulting from dilatation of sarcoplasmic reticulum terminal cisternae (SR-TC). In addition, disorganization of the sarcomeres and myofibrils, rupture of distended myofilaments, and mitochondrial damage accompanied the alterations. T-tubules were unaffected suggesting they are not the source of dilated vacuoles. Some differences about the onset and time-course of the alterations, as well presumptive hypotheses to explain the mechanisms involved in the dilatation of SR-TC, and abnormal mitochondria are discussed. All the morphological alterations were prevented by pre-treatment with TTX. We postulate, therefore that these abnormalities result from an increase in sodium influx into cell as a result of sodium channel activation by veratrine.

Key words: mitochondria/ sarcoplasmic reticulum/ skeletal muscle/ sodium channels activator/ transmission electron microscopy/ veratrine

Introduction

Several toxins which cause persistent sodium channel activation have been reported to produce alterations in the structure of nerves and/or muscles fibers. Albuquerque *et al.* (1971; 1973) and Warnick *et al.* (1971) in their studies on batrachotoxin (BTX) showed that this toxin induces ultrastructural changes in the muscle fibers, mainly in the sarcoplasmic reticulum, and at larger concentrations, in the neuromuscular junction. The effects on the muscle fiber structure were prevented by tetrodotoxin (TTX). The crude venom of the South American spider *Phoneutria nigriventer*, and its toxic fraction PhTx2, both of which induce persistent activation of the sodium channel (Fontana and Vital Brazil, 1982; 1985) was also found to produce morphological changes in mouse muscle and nerve after intramuscular (i.m.) or intraneural (i.n.) injection (Brook *et al.*, 1984; 1986; Cruz-Höfling *et al.*, 1985, Love *et al.*, 1986, Love and Cruz-Höfling, 1986) and in *in vitro* preparations (Matiello-Sverzuta and Cruz-Höfling, 2000). As with BTX, the changes could be prevented by TTX. Crotamine, a basic polypeptide toxin from the venom of South American rattlesnake *Crotalus durissus terrificus* (var. *crotaminicus*) and the chemically and pharmacologically related myotoxin *a* from the venom of the North American prairie rattlesnake, *C. viridis viridis*, are persistent activators of the sodium channel (Pellegrini, 1976; Pellegrini *et al.*, 1978; Chang and Tseng, 1978; Hong and Chang, 1985), and their deleterious effects on muscle fiber structure have been reported (Ownby *et al.*, 1976; Cameron and Tu, 1978; Ownby, 1982). Swelling of sarcoplasmic reticulum (SR) is the first step in myonecrosis caused by myotoxin *a* (Tu and Morita, 1983; Volpe *et al.*, 1986), what suggests SR as a putative shock organelle for their effects on skeletal muscle fibers. Ownby *et al.* (1976) reputed the changes to inhibition of Na^+K^+ATPase, and

consequent accumulation of ions within the cell followed by water, leading to swelling and eventually death of the fibers.

Besides BTX, others naturally occurring alkaloids such as, veratrine, veratridine, aconitine, grayanotoxin also lead Na^+ channels from excitable membranes to stay open longer (Hille, 2001). Veratrine is an extract of *Schoenocaulon officinale* seeds. Its main constituents are the ester alkaloids cevadine and veratridine (Benforado, 1967). This last compound has been shown to induce persistent activation of the sodium channel (Catterall, 1980). Veratrine, as expected, exerts the same or a similar action, as for veratridine (Vital Brazil and Fontana, 1985; 1993). As with BTX and *P. nigriventer* venom, veratrine affects sarcoplasmic reticulum with vacuolation of terminal cisterns (SR-TC), and in addition causes mitochondrial damage, hypercontraction of sarcomeres and fragmentation of myofilaments in a number of fibers, while others remain unaltered. The changes could be prevented by TTX (Cruz-Höfling *et al.*, 2002; Freitas *et al.*, 2001). SR seems to be one of the primary target of the alkaloid action in our model, and presumably the sarcomeric changes that follows, as distortion of myofibrils and fragmentation of myofilaments, result from vacuolar-induced deformation of SR. It was inferred that theses changes were caused by osmotic unbalance resulting from higher influx of Na^+ inside the fiber that brings together fluid and alters cell volume. However, it was not clear how the osmotic unbalance achieves SR.

Recently we proved that mitochondria are one target for veratrine. In rat isolated skeletal muscle mitochondria (RMM), veratrine caused a dose-dependent inhibition of respiration (Freitas *et al.*, 2006a) and decreased electron chain complexes enzymes activities (Freitas *et al.*, 2006b).

In the present study, we combined *in vitro* and *in vivo* studies in the investigation of ultrastructural effects of sodium overload in four types of skeletal muscles from mammalian and fish: the vital ventilatory diaphragm and the peripheral, non-vital soleus and EDL, both from mice and the lateral muscle from fish, which differ to each other in their contractile, fiber-type composition and metabolic properties. Sodium overloading was induced by veratrine which activates voltage-regulated sodium channels present in the surface of skeletal muscle fibers and peripheral nerves. The effects of veratrine incubation in mouse phrenic nerve-hemidiaphragm muscle preparations (5 and 10 μg/mL) were compared with the effects of i.m. injection on mice soleus and *extensor digitorum longus* muscles and in fish lateral muscle (10 μg/mL). In addition, the effects of veratrine on muscle contraction were studied in phrenic nerve-diaphragm *in vitro* preparations using the same toxin concentrations (5 and 10 μg/mL) as these employed in the morphological investigation.

Material and Methods

Chemicals

Veratrine was purchased from British Drug Houses Ltd (Poole, Dorset, England) and from Sigma Chemical Co. (St. Louis, MO, USA). Before an experiment, the veratrine was weighted and dissolved in distilled water by adding 0.1 N HCl. The pH of the concentrated solution (0.5 mg/mL) was adjusted to about 7.0 with 0.1 N NaOH. Tetrodotoxin was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and MS 222, from Sandoz (Basel, Germany). The Tyrode solution used had the following composition in mM: NaCl 136.8, KCl 2.7, $CaCl_2$ 1.8, $NaHCO_3$ 11.9, $MgCl_2$ 0.25, Na_2HPO_4 0.3 and glucose 11.0.

In vitro studies

Adult male Swiss white mice (20-25 g) were sacrificed by cervical dislocation and their diaphragms were immediately removed. The diaphragms and their nerves were horizontally mounted with the innervated (thoracic) surface facing upwards in a Petri dish containing Tyrode solution at room temperature (25°C) and aerated with carbogen (95% O_2, 5% CO_2).

For morphological studies phrenic nerve-diaphragm preparations taken from 30 mice were divided into: controls groups (G-I, -II, -V) and veratrine-treated diaphragm (G-III, -IV).

G-I (n=2) - Time zero controls: following excision, the diaphragms were immediately processed for transmission electron microscopy (TEM).

G-II (n=6) - The preparations were incubated in Tyrode solution alone for 15, 60 or 120 min.

G-III (n=12) - The preparations were incubated with either 5 or 10 µg/mL of veratrine for 15, 60 or 120 min.

G-IV (n=8) – Diaphragms were pre-incubated with 1 µg/mL of TTX for 15 min and, after rinsing with saline, were incubated with veratrine (5 or 10 µg/mL) for 60 or 120 min.

G-V (n=2) – Control preparations which were incubated with TTX alone (1 µg/mL) for 15 min.

After incubation, the Tyrode, veratrine or TTX solutions were removed and replaced with a freshly prepared Karnovsky fixative solution containing 3% glutaraldehyde (v/v) (Merck, Darmstadt, Germany) and 1% paraformaldehyde (p/v - Sigma) in 0.1 M sodium cacodylate buffer (Sigma) (pH 7.2 – 7.4) at 4°C. Selected areas of the diaphragm around the phrenic nerve branching known to contain a higher density of neuromuscular junctions were dissected out under a stereomicroscope. Muscle fragments (1 – 1.5 mm) of the sampled areas were transferred to fresh Karnovsky fixative for 12-72 h at 4°C and post-fixed in 1% OsO_4 in the same buffer for 1 h. After overnight block staining with 0.5% aqueous uranyl acetate and dehydration in an ethanolic series, the material was embedded in Araldite CY 212 (Cargill-Polysciences, Warrington, PA, USA). Thin (1 µm thick) sections stained with toluidine blue were examined by light microscopy (LM) in order to select areas of the diaphragm with small nerves and to count affected and unaffected muscle cells. From these areas, ultrathin (60 - 90 nm) were cut using Porter-Blum MT-2 and LKB-supernova ultramicrotomes fitted with either glass or diamond knives. The sections were collected on appropriate copper grids (200 mesh) and stained with lead citrate. Examination of the samples thus prepared was carried out using a Zeiss EM9S2, transmission electron microscope (TEM).

The effect of veratrine on muscle contraction was also studied in the mouse phrenic nerve-diaphragm preparation. Hemidiaphragms from six adult male Swiss white mice (25 – 30 g) were suspended in a 10 mL organ bath containing Tyrode solution at 37°C oxygenated with 95% O_2, 5% CO_2. Nerve stimulation was carried out with single maximal pulses of 0.2 ms duration. Direct muscle stimulation was performed with single pulses of 80 V and 2.0 ms. Contractions were registered on an E&M Physiograph using a Linear Core F50 isometric transducer. The stimuli were delivered by a Grass S48 stimulator.

In vivo studies

Mice

Adult male mice BALB/c (20-25 g) were obtained from the university's Animal House Unit, 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*. Nine mice were anesthetized with sodium pentobarbital (40-70 mg/kg i.p. – Hypnol; Cristalia, São Paulo, Brazil) and SOL and EDL muscles were exposed for the *in situ* injection of 5 µl veratrine (10 µg/ml) using a Hamilton syringe. After 15, 30 and 60 min of veratrine injection (n=3 animals/time interval), the mice were sacrificed and the EDL and SOL muscles removed and processed for TEM.

Fish

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. The group of fish (n=9) received 10 µg/ml veratrine concentration intramuscularly (i.m.) at a central point in the lateral

musculature after anesthesia with tricaine methanesulphonate (MS 222, 1:100,000). Fifteen, 30 or 60 min later, the anesthetized animals (n=3 fish/time interval) were sacrificed by cervical dislocation and the muscle excised and processed for TEM.

Pre-treatment with TTX

Under effect of anesthesia, the animals, mouse or fish, received 1 µl of TTX (50 µM in sterile saline; Sigma), i.m., 5 min before the administration of veratrine (n=3/group). After 30 min of alkaloid injection, the muscles were excised and processed for TEM.

Control groups (mouse and fish)

Control animals (n=3/group) received an injection of 0.9 % sterile saline solution under the same experimental conditions used for veratrine-treated groups.

Ultrastructural studies

The mice and fish muscles fragments (1-2 mm) were immersed in Karnovsky's fixative (2.5% glutaraldehyde plus 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4) for overnight. Posteriorly, the muscles fragments were post-fixed in buffered 1% OsO_4, 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 LM. Ultrathin sections (60-70 nm thick) were stained with lead citrate and uranyl acetate, and examined in a LEO 906 TEM.

All the procedures with animals were done within the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Semi-qualitative analysis

An estimate of the number of damaged and undamaged muscle fibers of the mice muscles and fish lateral musculature (n=40/section) incubated and injected with veratrine taken by counting the profiles of cells in 10 toluidine blue-stained 1 μm thick sections were made.

RESULTS

Morphological changes

Mouse diaphragm muscles

In diaphragms processed at time zero (Group I) and in those incubated with Tyrode solution alone (Group II), the ultrastructural features were normal in appearance. In longitudinal sections, the sarcomeres extending between two consecutive Z lines were well-dimensioned with a correct spacing of the myofilaments, as demonstrated by the presence and regular arrangement of all the major bands (A, I and H) in the muscle fiber. Both the intermyofibrilar mitochondria and the sarcoplasmic reticulum aligned along the myofibrils (Fig. 1A).

In periods as short as 15 min of veratrine incubation diaphragm showed no vacuoles in myofibers of mice muscles (Fig.1B). However, at both 60 and 120 min incubation, in the affected muscle cells, the most striking effect was the disturbance of the SR morphology. Distended and swollen vacuoles, sometimes as large as two or three sarcomeres, were present in the damaged sarcotubular system (Fig. 1C-D). The oversized SR cisternae induced curving of the myofibrils although the sarcomeres retained their normal size. In addition, the A, I and H band as well as the Z and M lines appeared intact (Fig. 1C-D).

Despite the impressive vacuolization of the SR cisternae, the integrity of the SR membranes was unaffected. The mitochondria showed no visible morphological abnormality in these degenerated muscle fibers (Fig. 1C-E).

Within the 60 or 120 min incubation of the diaphragm with veratrine, some transverse tubules could not be readily identified or to be distinguished from the fragmented vacuoles randomly scattered within the degenerated muscle cell (Fig.1C).

The morphological changes following muscle incubation with veratrine were qualitatively alike. In addition, there were no significant differences in responses produced by either 5 or 10 μg/mL of veratrine.

Mouse EDL muscle

The saline control EDL exhibited normal ultrastructural organization with well-defined sarcomeres and triads were located in the limit between A and I bands. Mitochondria, SR-TC and T-tubules were morphologically preserved (Fig. 2A).

After 15 min of veratrine injection, some muscle fibers showed dilatation of SR-TC and swollen mitochondria (Fig. 2B). At 30 min of veratrine injection, hypercontracted fibers with disorganization of sarcomeres, and dilated SR-TC, and normal-architectured fibers, but presenting degenerated mitochondria without cristae and electronlucent matrix were seen (Fig. 2C,D). T-tubules, though presented intact morphology after injection of alkaloid (Fig. 2D). At 60 min post-injection (p.i.) of veratrine, focal areas with ruptured myofibrils and swollen mitochondria were seen in a few muscle fibers. T-tubules remained intact (Fig. 2E).

Mouse SOL muscle

As for EDL, longitudinal electronmicrographs of saline-injected SOL muscle showed regular sarcomeres and high concentration of mitochondria located underneath cell membrane and among myofibrils (data not shown).

At 15 min veratrine p.i., many muscle fibers already had swollen and damaged mitochondria (Fig. 3A), differently from EDL muscle. At 30 min, affected fibers included swollen mitochondria, dilated SR-TC, which together with fiber hypercontraction and disappearance of sarcomere bands hindered distinguishing vacuoles born from SR or T-tubules (Fig. 3B). After 60 min, the majority of the morphological alterations disappeared but edema persisted on sarcolemmal mitochondria (not shown).

Tilapia fish muscle

The lateral musculature of saline-injected *O. niloticus* fish exhibited well-aligned sarcomeres and myofibrils. Triads were arranged at Z-lines and a well-developed sarcotubular system is exhibited (Fig. 4A). In fish injected with veratrine, just moderate disorganization of sarcomeres without any abnormality in regard to SR and mitochondria was seen after 15 min of veratrine injection (data not shown). However, at 30 min p.i., the affected fibers showed swelling of SR-TC, rupture of myofilaments (Fig. 4B), swollen irregular in shape mitochondria, and less definition of sarcomere bands (Fig. 4C). T-tubules remained unaltered (Fig. 4C,D). At 60 min, the proportion of affected muscle fibers decreased, remaining only rupture of myofilaments and swelling of mitochondria on focal areas. T-tubules remained preserved (Fig. 4E).

Pre-treatment with TTX

Pre-treatment with TTX (1 µg/mL) prevented ultrastructural alterations induced by veratrine in all the muscles tested, principally the disorganization of sarcomeres, rupture of myofilaments and dilation of SR terminal cisternae on mouse diaphragm, EDL and SOL

muscles (Fig. 5A) and fish lateral muscle (Fig. 5B). However, sarcomeres did not achieve the regular size of about 2.5 µm, remaining about 40% shortened.

The mouse diaphragm muscle incubated with TTX alone (control Group V) showed normal morphology similarly to other control groups (Group I and II) (data not shown).

Semi-qualitative analysis

The estimate of veratrine-affected fibers taken from 1 µm thick plastic sections stained with toluidine blue and examined by LM showed an average of 30 – 35% after 60 min of veratrine treatment for mice diaphragm and EDL. In mouse soleus and fish lateral muscle muscles an approximate equal proportion of affected fibers were counted at 30 min treatment, but this was reduced to about 5% at 60 min.

Effects on muscle contraction

Veratrine in the same concentrations as those used in the ultrastructural study (5 and 10 µg/mL) induced the typical effects of strong veratrinization (Krayer & Acheson, 1946), namely, a smooth rise in tension to a maximum several fold higher than that of the normal followed by a long, slow return to the base line level (Fig. 6). In one of the six experiments, however, the response 15 min after the addition of 5 µg/mL of veratrine to the bath was that of weak veratrinization: a twitch-like initial contraction and partial relaxation followed by a secondary rise and a slow relaxation (Fig. 6I-B). Responses to nerve stimulation were blocked within 30 and 60 min after addition of 5 and 10 µg/mL of veratrine, respectively (Fig.6). After

blockade of the contractions elicited by indirect stimulation, the diaphragm still responded to direct muscle stimulation and the myograms always demonstrated strong veratrinization.

These experiments demonstrate that the concentration of veratrine used in the ultrastructural study should be considered large (5 μg/mL) and very large (10 μg/mL) based on their effect on muscle contraction and intramuscular nerve conduction.

DISCUSSION

Our initial experiments were designed to select the optimal treatment conditions based on morphological/ultrastructural parameters. We chose the mitochondria, which give the first signs of physiological stress, and the organizations of the muscle sarcomeres as the two best indicators of structural damage. As clearly shown in figures, there were no adverse morphological/anatomical changes in any of the control preparations during the course of the experiments. We therefore concluded that the conditions we had selected were adequate for our purposes.

A range of 65 to 70% muscle fibers from mice diaphragm and EDL muscles (60 min exposure to veratrine) and mouse soleus and fish lateral musculature (30 min exposure to veratrine) showed normal morphology. (Mouse soleus and fish lateral muscle had partial and spontaneous recovery of the changes at 60 min p.i.). With 60 min after veratrine exposure this proportion in about 5%. These fibers were intermixed with affected ones where the most prominent change observed was a disturbance of the SR structure with swollen vacuoles along the length of the sarcomeres. The huge inflation of SR-TC seems to be largely responsible for the disorganization and eventual dissolution of the normal sarcomere architecture. It is also possible that disruption of mitochondria or alteration of its morphology could be linked to alterations initiated in the SR, in the three types of muscle, except the diaphragm. In the mouse phrenic nerve-hemidiaphragm muscle preparations, the mitochondria and sarcomeres appear to be unaffected by these sarcotubular morphological disturbances. This difference among diaphragm, which is a vital muscle and the other three peripheral, non-vital muscles, is one more to be added to the vast list of differences of diaphragm. The diaphragm is a mixed

muscle with fast- (20%) and slow- (60%), and intermediate-contracting (20%) fibers and a high oxidative capacity (Padydula and Gauthier, 1970), which enroll the muscle as highly fatigue-resistant and able to continuous activity throughout life (Itoh et al. 2004). On the other hand, EDL has predominantly fast-contracting fibers, works under glycolytic metabolism, and has low-resistance to fatigue. Soleus has predominantly slow-contracting fibers and works with oxidative metabolism (Ellisman *et al.*, 1976; Takehura *et al.*, 1994). Both muscles work discontinuously, differently from diaphragm. Lateral muscle of fish is composed of red muscle formed by slow-contracting oxidative fibers at the periphery, close to fins, by mixed oxidative and glycolytic fibers in the intermediate portion, and by white muscle with fast contracting glycolytic fibers in the deep portion of the muscle (Mascarello *et al.*, 1986). The resistance to damage shown by the mitochondria of mouse diaphragm can be accounted for the remarkable capacity of responding even under adverse conditions as chronic hypoxia, hypercapnia, and neuromuscular blocking drugs, among others, while peripheral muscles are no longer responsive (El-Khoury *et al.*, 2003; Itoh *et al.*, 2004). The study of the time-course of development of vacuoles formation in curarized diaphragms incubated with 5 μg/mL of veratrine at 10 and 20 min, showed intact mitochondria and T-tubules but SR cisternae in progressive but incipient state of swelling (Cruz-Höfling, M.A.; Fontana, M.D. and Vital Brazil, O., unpublished work). Vacuolation due to the swelling of T-tubules, if existing in the periods observed (60 or 120 min) seems to be secondary to SR vacuolation and represent later stages of the muscle contact with veratrine. Enlargement of the T-tubular system experimentally obtained by Casademont *et al.* (1988) in transsected rat *soleus* muscle *in vitro* can be prevented by Na^+-free medium of incubation, by incubation at 10°C, by media containing ouabain or 2-4 dinitrophenol, or if the muscle ends were not transsected. The

authors concluded that the vacuoles result from the active influx of Na^+ within the muscle cells trough their cut ends by the activity of the Na^+-K^+-ATPase.

When veratrine was applied to the incubation bath or injected after pre-treatment with TTX (1 μg/mL) no morphological vacuolar alterations occurred, except shortening sarcomeres. This clearly demonstrate that the veratrine-induced ultrastructural changes may be ascribed to Na^+ channel activation and to the consequent increase in intracellular Na^+ concentration and water, as suggested by Ownby (1982).

Other toxins that activate the sodium channel are reported to induce similar alterations involving the SR. BTX, a complex steroid alkaloid from the skin of the Colombian poison-dart frog *Phyllobates aurotaenia* (Dendrobatidae), is one of the most potent toxins known to man. BTX toxicity was shown to result from its ability to activate the sodium channel (Warnick *et al.*, 1971). Its effect on muscle fiber ultrastructure was studied in the isolated diaphragm and *extensor digitorium longus* of the rat where it was found that BTX, at 0.0025 – 0.08 μg/mL, induces vesiculation of the terminal cysternae (TC) and of the longitudinal part of the SR (Albuquerque *et al.*, 1971). In concentrations equal or greater than 0.005 μg/mL it also causes disruption of the TC and SR. No alterations in the muscle fibers were induced by BTX in the presence of TTX (2 μg/mL).

Crotamine from the venom of *C. durissus terrificus* (var. *crotaminicus*) and myotoxin *a* from that of *C. v. viridis* are basic, low toxicity polypeptide of 4.9 and 4.1 kDa respectively. They are chemically (Tu, 1982), immunologically (Ownby, 1982) and pharmacologically related. Both toxins activate sodium channels (Pellegrini, 1976; Pellegrini *et al.*, 1978; Chang and Tseng, 1978; Hong and Chang, 1985) and induce repetitive muscle responses (Vital Brazil *et al*, 1979; Hong and Chang, 1985). The morphological muscle changes induced by myotoxin

a were studied by Ownby and coworkers (Ownby, 1982; Ownby *et al.*, 1976). They found focal lesions containing areas of partial vacuolization, which though progressed with time post toxin injection, culminating in marked dilatation of the SR and perinuclear space followed by fragmentation of SR huge vacuoles into numerous smaller vesicles. The mitochondria were slightly swollen and lacked cristae. Intact T-tubules were present in these areas and the sarcolemma and basal lamina of necrotic muscle fibers were intact. The preventive effect of TTX on the myotoxin *a*-induced lesions was not investigated. The authors attributed to Na^+-K^+-ATPase inhibition a role in the appearance of vacuolization. The effect of crotamine on the skeletal muscle fiber was examined by light microscopy after intramuscular injection in mice and revealed vacuolization of the skeletal muscle fibers, qualitatively the same as that induced by myotoxin *a* (Cameron and Tu, 1978). The above studies, in conjuction with the present work, thus show that veratrine, BTX, myotoxin *a* and crotamine produce essentially similar morphological alterations in the skeletal muscle fiber (the small discrepancies observed may be attributed to differences in experimental procedures and muscles examined). The sodium influx which the opening of the sodium channel promotes carries water into the muscle fibers, particularly into the SR where the sodium ions that penetrate the cell seem to concentrate. Using isolated nephrons, Ginn and coworkers (1968) studied the effect of intracellular sodium accumulation produced by ouabain-induced inhibition of the Na^+-K^+-dependent ATPase and observed that the first utrastructural change was also dilatation of the endoplasmic reticulum. How the sodium ions penetrate into these structures to attain a concentration most likely greater than that in the cystosol is not clear. Following the authors, the dilatation of the SR and of the endoplasmic reticulum suggests that these structures may play a role in attenuating the total cell swelling induced by increased intracellular Na^+ or K^+ concentrations. Ladka and Ng

(2000) showed that non-toxic concentration of veratridine greatly increased expression of the α_2 Na^+-K^+-ATPase isoform in cultured C2C12 skeletal muscle cells, a cell line derived from mouse thigh muscle whereas Taormino and Fambrough (1990) showed that expression of α_1 isoform is upregulated by veratridine. Both in rat skeletal muscle cells, as well as in cultured chicken muscle cells, TTX treatment caused a significant decline in the number of Na^+-K^+-ATPase (Brodie and Sampson, 1989) and reverses the upregulation of the enzyme induced by veratridine (Wolitzky and Fambrough, 1986). Na^+-K^+-ATPase is an integral membrane protein responsible for the active transport of sodium and potassium ions across the plasma membrane and maintenance of Na^+ and K^+ electrochemical gradient through cell membrane (Harrison *et al.*, 1997; Nielsen and Clausen, 1996; Nielsen and Overgaard, 1996). Skeletal muscle contains the largest percentage of Na^+/K^+ pump and the major pool of K^+ in the body (Clausen and Everts, 1989). Changes in expression and/or in activity of the Na^+-K^+ pump in skeletal muscle affect metabolism of K^+, which in turn alter the function of the muscle (Harrison *et al.*, 1997, Nielsen and Clausen, 1996; Nielsen and Overgaard, 1996). Disturbances of physiology of Na^+-K^+-ATPase and homeostasy loss may contribute to changes in SR volume.

We also speculate that the formation of SR-TC vacuoles and mitochondria swelling caused by veratrine would be through the increase in calcium influx via the reversed-mode of Na^+/Ca^{2+} exchange, secondary to higher Na^+ influx (Cruz-Höfling *et al.*, 2002), caused by iterative despolarization (Fontana and Vital Brazil, 1985), thus elevating intracellular calcium. In this situation one Ca^{2+} released is exchanged by entry of three Na^{2+}. Evidences indicating the existence of Na^+/Ca^{2+} exchange in mouse intact single skeletal muscle fibers were reported by Balnave and Allen (1998). On the other hand, Na^+ channel-activating toxins, as veratrine, once fitted into the open channel pore, interact with all their four inner helices, what

influences the structure of the ion permeation pathways probably including the selectivity-filter region. To affect both conductance and selectivity, the activator should bind like a bridge between the gate and the selectivity filter. Under the circumstance activated channels lose their selectivity (Tikhonov and Zhorov, 2005). From this it could be understandable that the opened channel becomes permeable to the entrance of other cations, therefore promoting increased osmotic pressure and so fluid influx, also provoking loss of cell and organelle homeostasy.

Endo and Nakajima (1973) explained the swelling of SR in skinned skeletal muscle fibers incubated with hypertonic solutions by ionic replacements that would alter SR membrane permeability inducing consequently a release of calcium of SR terminal cisternae, without altering the T system selectivity. The replacement of less permeant anions with chloride could cause swelling of SR terminal cisternae, since chloride ions would enter the lumen of SR along with other cations and water (Endo, 1977).

Beyond its action in SR, the veratrine interferes on mitochondrial bioenergetics. Our recent study showed that this alkaloid causes a dose-dependent inhibition on oxygen consumption and dissipation membrane electrical potential supported by NADH and $FADH_2$-linked substrates in isolated rat skeletal muscle mitochondria (Freitas *et al.*, 2006a). Moreover, we demonstrated that veratrine decreases nicotinamide adenine dinucleotide dehydrogenase (NADH), succinic dehydrogenase (SDH) and cytochrome oxidase (COX) activities and induces ultrastructural changes on isolated mitochondria (Freitas *et al.*, 2006b). We suggested that veratrine could act on mitochondrial membrane phospholipid configuration, explaining the morphological, biochemical and cytochemical alterations seen on isolated mitochondria, and here in in vivo studies on mouse EDL and soleus muscle and fish lateral muscle. An

interesting point to investigate is the bioenergetics from isolated diaphragm muscle mitochondria incubated with veratrine or veratridine.

Our results indicate that Na^+ overload induced by veratrine alters osmotic balance in skeletal muscle fibers by a mechanism that needs investigation. A number of viable possibilities may account for the osmotic disturbances seen in SR morphological alteration. As the mitochondria and SR are buffering cellular organelles, they are the principal structures affected when ionic disturbances within the cell occur. Transient changes as those observed in soleus and tilapia muscle, that show partial regression at 60 min, are typical of osmotic unbalance.

Recently, Hu and colleagues (2003) showed that Na^+ and K^+ ions interact with ryanodine receptors located in SR terminal cisternae. During excitation-contraction coupling, a change of Na^+ and K^+ levels occurs in the narrow T-tubules-SR gap when the action potential propagates into the T-tubules membrane (Fitts and Balong, 1996). Na^+ entry may positively modulate the cooperation between ryanodine receptors and increase the release of calcium ions from the SR (Hu *et al.*, 2003).

Ionic disturbances in the cell certainly will affect metabolism of calcium in SR and mitochondria. The existence of evidences indicating that there was cross-talk between those two cell organelles regarding calcium transport, more likely entitled them for the action of veratrine. The effects generated by veratrine, an activator of sodium channels, in different muscle types (from mammalian and fish) veratrine, and their abolishment by TTX, a blocker of sodium channels seem to support the ionic disturbances as the cause of ultrastructural changes affecting mitochondria and sarcoplasmic reticulum. The mechanisms responsible for

these changes, particularly an explanation of how fluid is transported to SR-TC needs to be clarified.

Another interesting point to be investigated is why several muscle fibers were morphologically unaffected during incubation. It can be speculated that the clue for the question could rest in the number of sodium channels, development of T-system and sarcoplasmic reticulum which differ in the different fiber types belonging to diaphragm, soleus, EDL and fish lateral muscle. Also the well-known existence of physiologically and biochemically different subpopulations of mitochondria can be involved in these effects.

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LEGEND OF FIGURES

FIGURE 1: Electron micrographs of mouse diaphragm muscle fibers. **(A)** Control: incubated with Tyrode solution for 60 min (Group II). Note the intact structure of the sarcomeres (S), the Z lines, bands A, I and H, mitochondria (mi) and sarcoplasmic reticulum (SR); **(B)** Muscle incubated with 10 µg/mL veratrine for 15 min: sarcomeres, sarcoplasmic reticulum cisternae (SR), T-tubules (t) and mitochondria (mi) are normal morphology; **(C-E)** Muscle incubated with veratrine for 60 min: hypertrophied cisternae of the sarcoplasmic reticulum (SR) sometimes reaching two or three sarcomeres in size. The distorted sarcomeres maintain intact structural components (lines and bands). Mitochondria appear normal (mi). T-tubules are intact (t) (**D**,**E**). Bars= 1 µm (A, B, D, E) and 0.5 µm (C).

FIGURE 2: Electron micrographs of mouse EDL muscle fibers. **(A)** Control: normal fiber morphology; **(B)** Fifteen min after injection of 10 µg/mL veratrine: observe large vacuoles resulting from swelling of SR terminal cisternae (SR) and swollen mitochondria (*); **(C-D)** Thirty min post-injection: zones of hypercontraction (h) are seen on muscle fibers **(C)**. Note a desorganization of the myofibrils due to numerous vacuoles from SR. In some muscle fibers, presence of degenerated (d) mitochondria **(D)**; **(E)** Sixty min post-injection: focal areas with ruptured (r) myofilaments in some muscle fibers. Mitochondria (mi), SR cisternae and T-tubules (t) are preserved. Bars= 1 µm for all figures.

FIGURE 3: Electron micrographs of mouse SOL muscle fibers. **(A)** Fifteen min after injection of 10 μg/mL veratrine: muscle fiber with well-defined sarcomeres and degenerated (d) and swollen mitochondria; **(B)** Thirty min post-injection: presence of dilated cisternae from SR and swollen (*) mitochondria on hypercontracted muscle fibers. Note normal T-tubules (t). Bars= 1 μm for all figures.

FIGURE 4: Electron micrographs of lateral musculature from *O. niloticus* fish. **(A)** Control: typical organization of muscle fiber with well-aligned sarcomeres and myofibrils. SR cisterns and T-tubules (triads) are normal appearance; **(B-D)** Thirty min after injection of 10 μg/mL veratrine: observe disorganization and rupture of myofibrils. Presence of dilated SR cisternae (**B** and **D**) and swollen (*) mitochondria **(C)**. In higher magnification **(D)**, micrograph showing intact T-tubules (t); **(E)** Sixty min post-injection: some regions of muscle fiber with presence of ruptured myofibrils and swollen (*) mitochondria. Bars= 1 μm for all figures.

FIGURE 5: Electron micrographs of mouse SOL muscle **(A)** and lateral muscle from *O. niloticus* fish **(B)** after injection of TTX (1 μg/mL) and veratrine (10 μg/mL). Note the preservation of structural sarcomeres and sarcotubular system by TTX. Bars= 1 μm for all figures.

FIGURE 6: Effect of veratrine on contractions of the mouse diaphragm elicited by supramaximal single pulses of 0.2 ms duration applied to the phrenic nerve and, after blockade, by direct muscle stimulation with single pulses of 80 V, 2 ms. **Upper traces (I):** 5

μg/mL of veratrine: A- Control; B and C- Response to indirect stimulation 15 and 30 min after veratrine addition to the bath; D- 60 min after veratrine, absence of response to indirect stimulation and a few min thereafter response (E) to direct muscle stimulation. **Lower traces (II):** 10 μg/mL of veratrine: A- Control; B- Response to indirect stimulation 15 after the addition of veratrine to the bath; C- 30 min after veratrine, absence of response to indirect stimulation; D and E- Response to direct muscle stimulation 30 and 60 min after veratrine (asterisks indicate responses elicited by direct muscle stimulation).

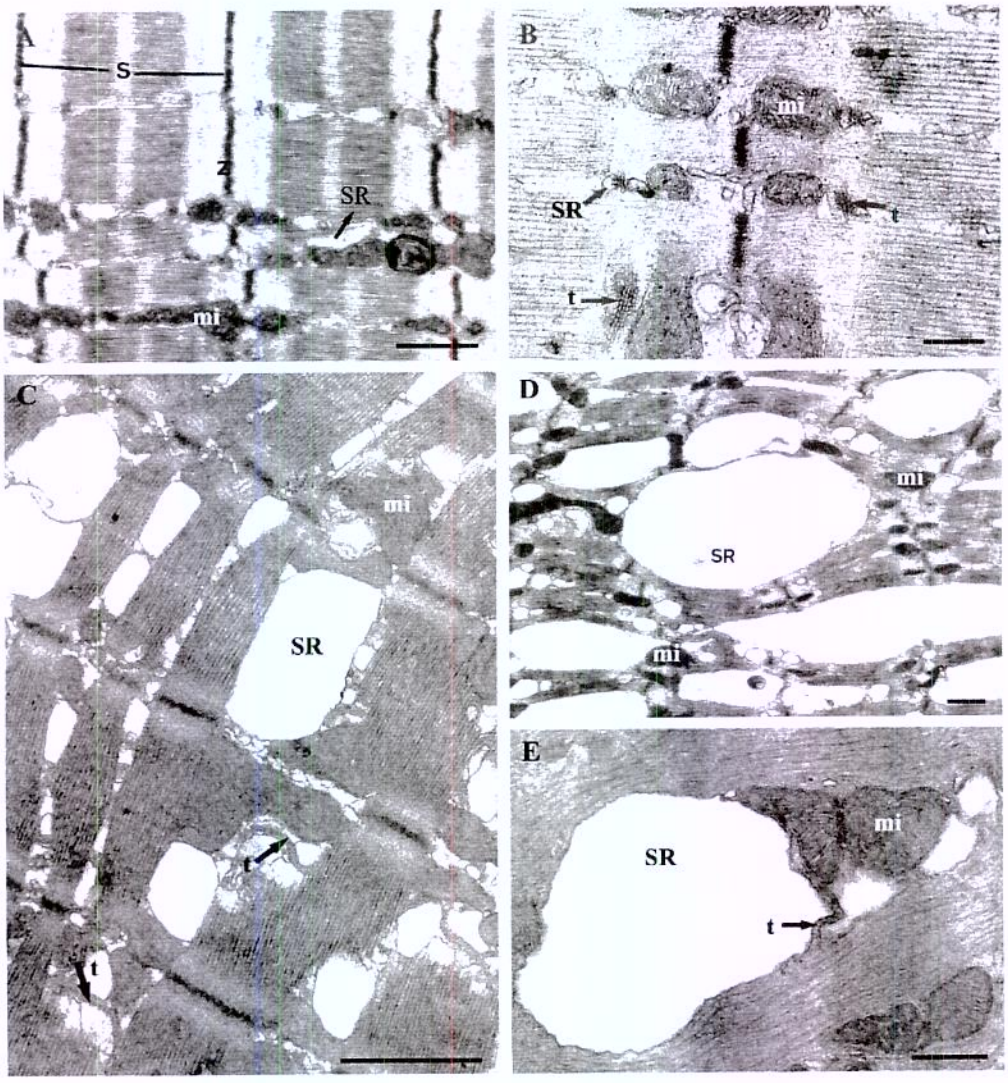

FIGURE 1
Cruz-Hofling et al., 2006

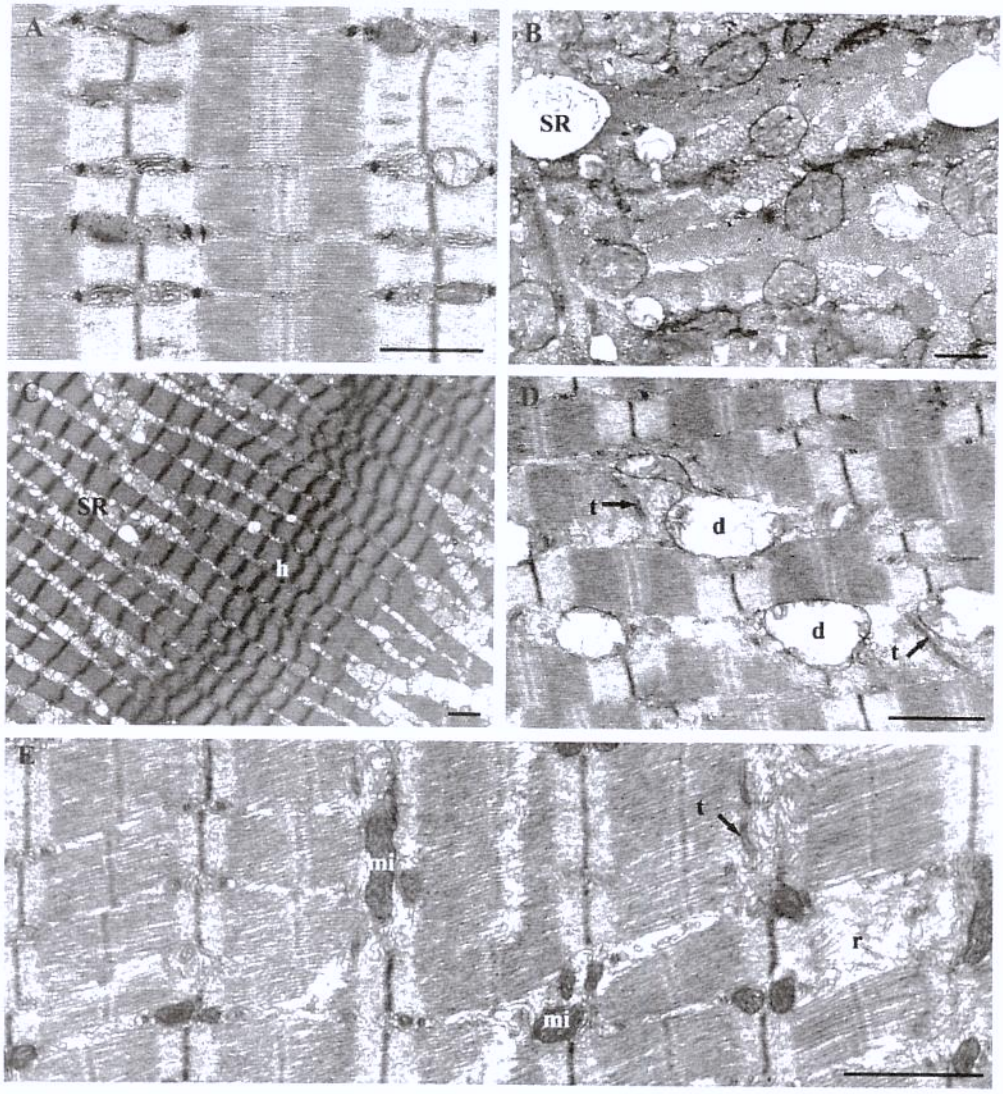

FIGURE 2
Cruz-Hofling et al., 2006

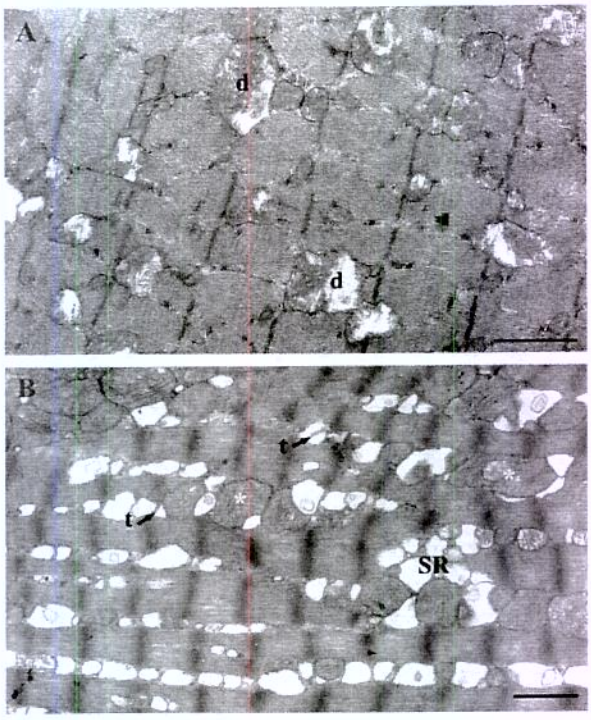

FIGURE 3
Cruz-Hofling et al., 2006

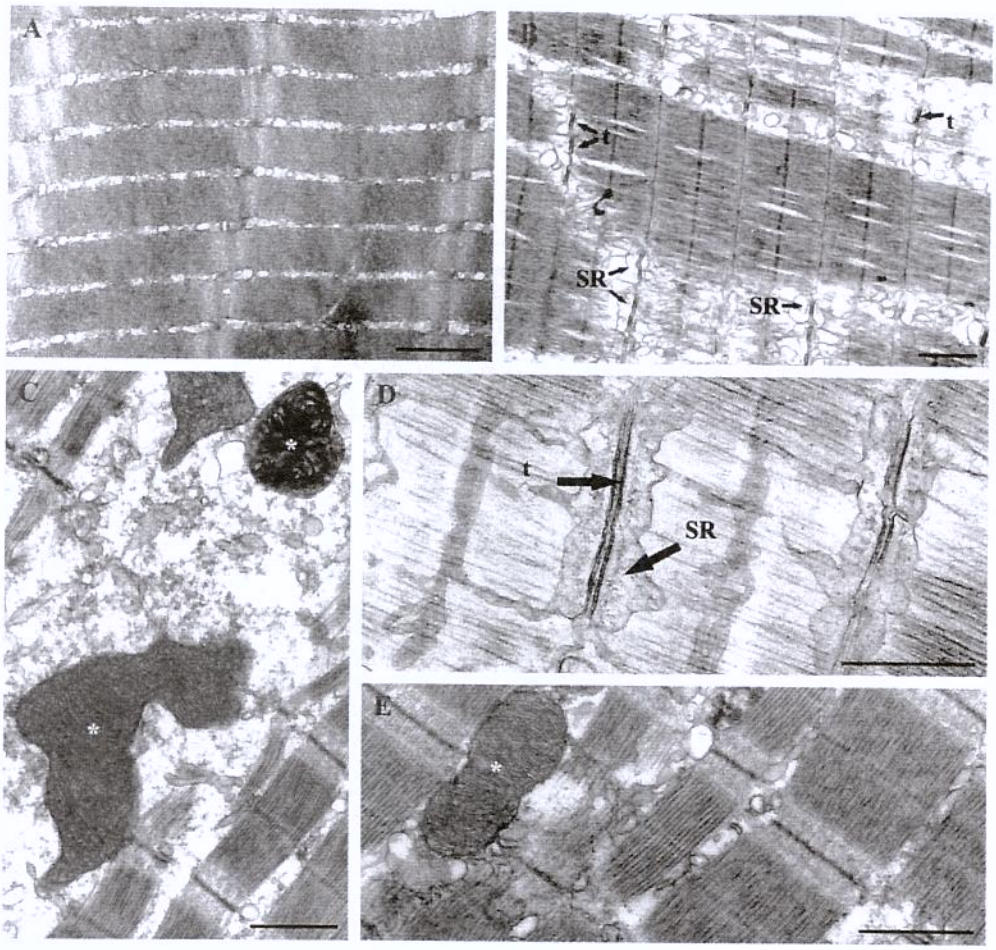

FIGURE 4
Cruz-Hofling, et al. 2006

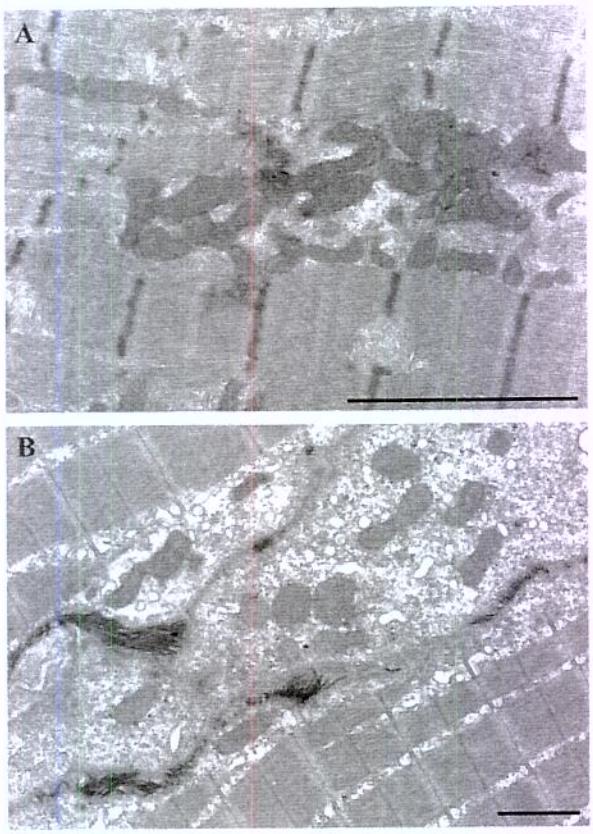

FIGURE 5
Cruz-Hofling et al., 2006

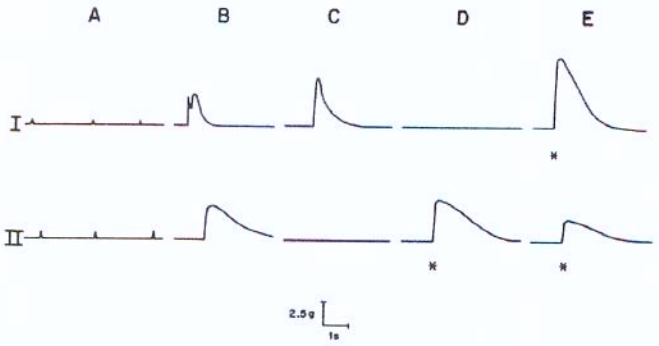

FIGURE 6
Cruz-Hofling et al., 2006

5. CONCLUSÕES FINAIS

- **MITOCÔNDRIAS**

Considerando que:

- *A veratrina e a veratridina, ambas toxinas ativadoras de canais de sódio voltagem dependentes, alteram o potencial elétrico transmembrana e inibem a atividade das enzimas dos complexos respiratórios nas mitocôndrias isoladas de músculo, porém essa ação não é verificada nas mitocôndrias isoladas de fígado.*

Concluímos que:

- o alcalóide e seu éster podem atuar na configuração fosfolipídica da membrana interna mitocondrial, e que essa ação é tecido-específica, ou seja, enquanto as mitocôndrias isoladas de músculos esqueléticos são susceptíveis aos efeitos tóxicos deste alcalóide, as mitocôndrias do fígado não mostram alterações no consumo de oxigênio nas concentrações utilizadas.

- existem populações mitocondriais cujas propriedades bioenergéticas possuem especificidades inerentes ao microambiente tecidual, o que explicaria a resposta das mesmas a veratrina e veratridina.

- uma vez que a veratridina é o principal éster alcalóide presente na veratrina (mistura de veratridina, cevadina e veracevina), e que pequenas concentrações da toxina foram necessárias para inibir o consumo de oxigênio, seriam a ela atribuídas as alterações morfológicas e bioquímicas induzidas nas mitocôndrias isoladas de músculos esqueléticos.

- *A veratrina, quando injetada no músculo (i.m.) EDL e sóleo de camundongos, e no músculo lateral de tilápia provoca alterações morfológicas nas mitocôndrias que se caracterizam por deformações no seu tamanho e perfil, na ruptura das cristas e membranas ou na maior densidade da matriz mitocondrial, vistas até 60 minutos após a injeção intramuscular do alcalóide. A ação tóxica da veratrina não foi observada nas mitocôndrias do músculo diafragma no período de 15 até 60 minutos de incubação. A ação da veratrina foi transitória nos experimentos in vivo, revertendo-se ao longo do período experimental, sendo a reversão mais precoce nas mitocôndrias do diafragma do que nos músculos periféricos. As alterações mitocondriais não se manifestaram quando houve pré-tratamento (i.m.) com tetrodotoxina, um bloqueador de canais de sódio, antes da administração da veratrina.*

Concluímos que:

- a ação da veratrina induzindo alterações estruturais nas mitocôndrias tem na sua raiz a alteração da fisiologia dos canais de sódio, que permite um maior influxo de sódio para a célula muscular (e presumivelmente de outros cátions) e por conseguinte maior pressão osmótica interna levando à entrada passiva de fluido para o interior. Essa dedução decorre do fato de que a administração prévia de TTX previne as alterações mitocondriais.

- as características bioquímicas, contráteis e metabólicas dos quatro tipos de músculos examinados são responsáveis pelas diferenças mostradas por eles a veratrina. O músculo diafragma, por ser um músculo vital, teria maior margem de segurança e, portanto suas mitocôndrias teriam maior resistência à ação da droga.

- as alterações morfológicas possam refletir tanto uma ação indireta da veratrina sobre as mitocôndrias, ou seja, o desequilíbrio iônico celular é que afetaria a fisiologia da organela, que se refletiria na sua morfologia, ou, haveria uma ação direta da toxina, que poderia entrar na célula e afetar a bioenergética da mitocôndria e a alteração da sua morfologia, ou ambas. Se a veratrina entra ou não na célula, se ela apenas se internaliza no poro do canal, ou se o canal ao permanecer aberto pela ação da veratrina perde sua seletividade e torna-se permeável a outros íons, são questões que necessitam ser esclarecidas.

- *A veratrina diminui a atividade das enzimas NADH-desidrogenase, SDH e COX nas membranas e cristas de mitocôndrias isoladas de músculos esqueléticos nas concentrações de 250 e 500 µg/ml, observadas através de métodos citoquímicos ultraestruturais. Além disso, houve uma tendência de aumento no tamanho das mitocôndrias após incubação com 250 µg/ml de veratrina, porém não foi significativo.*

Concluímos que a veratrina interfere diretamente na atividade das enzimas pertencentes aos complexos da cadeia respiratória mitocondrial, corroborando com os resultados de inibição da respiração observados nos ensaios bioquímicos.

- **RETÍCULO SARCOPLASMÁTICO**

Considerando que:

- *A ação da veratrina (i.m.) leva à grande dilatação das cisternas terminais do retículo sarcoplasmático (RS) nos músculos diafragma, EDL e sóleo de camundongos, e no músculo lateral de tilápia (teleósteo), sem alterar a morfologia dos túbulos-T, nas concentrações usadas e tempos observados em nosso modelo experimental. Deste modo, os vacúolos são decorrentes apenas da dilatação do RS. Essas alterações do RS são imediatas e espontaneamente reversíveis. Não afetam todas as fibras musculares do músculo. A reversão é mais precoce no diafragma de camundongos e no músculo lateral da tilápia. Nestes músculos é menos freqüente a completa perda da arquitetura dos sarcômeros por conta da alteração de volume das cisternas terminais do RS, do que no EDL e sóleo. A desorganização dos sarcômeros e miofibrilas, bem como a ruptura dos miofilamentos são secundárias à expansão sofrida pelas cisternas terminais. O pré-tratamento com TTX previne o aparecimento dos grandes vacúolos derivados das cisternas terminais aumentadas, porém o tamanho dos sarcômeros não atinge os valores convencionais, apresentando redução de cerca de 40% no seu comprimento.*

Concluímos que a ação da veratrina, quando injetada em músculos esqueléticos, ao aumentar a concentração osmótica intracelular, afeta também a permeabilidade e a seletividade da membrana desta organela, aumentando o influxo de cátions e água para as cisternas terminais,

justificando assim as alterações ultraestruturais observadas. A ação, porém parece ser seletiva, uma vez que a maioria das fibras musculares apresenta estrutura normal. Os vacúolos não representam os túbulos T dilatados, porque a tendência é a diminuição da concentração eletrolítica dentro deles (exterior da célula) e não o contrário. O mecanismo pelo qual à sobrecarga de sódio dentro da célula e a osmolaridade aumentada levam ao aumento do tamanho das cisternas terminais do RS é desconhecido, havendo, porém várias hipóteses que precisam ser avaliadas. A dilatação do retículo parece ser um recurso da célula para aliviar o aumento de volume celular decorrente da perda da homeostasia.

Conclusão Geral: Os efeitos da veratrina sobre mitocôndrias e o retículo sarcoplasmático mostram que a ação tóxica do alcalóide em diferentes sistemas biológicos pode envolver outros mecanismos de ação, além da ativação de canais de Na^+-dependentes de voltagem. O aumento da concentração de íons sódio intracelular induzido pela veratrina causa um distúrbio osmótico na fibra muscular, e na tentativa de reverter este efeito deletério para a célula, as mitocôndrias e o retículo sarcoplasmático agem conjuntamente como "organelas tamponantes", refazendo o equilíbrio hidroeletrolítico nas fibras. Deste modo, estas organelas seriam as principais estruturas alvo da ação tóxica da veratrina.

Concluímos que a veratrina e a veratridina podem ser ferramentas úteis para estudo dos mecanismos fisiopatológicos envolvendo disfunções mitocondriais e do retículo sarcoplasmático. Uma vez que a veratrina causa respostas iterativas na membrana muscular, ela pode também ser ótima ferramenta para investigar o transporte de cálcio entre as duas organelas tampões da célula, as mitocôndrias e o retículo sarcoplasmático, durante o acoplamento excitação-contração.